Investigating the use of standardized EuroFlow™ panels for the characterisation and diagnosis of Chronic lymphocytic leukaemia in the Tygerberg Academic Hospital, South Africa.

By

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Declaration

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Abstract

Background: Flow cytometry (FC) immunophenotyping is crucial in the diagnosis and classification standardisation of haematological malignancies. FC techniques require standardisation to produce reliable and reproducible results which are important in inter-laboratory studies for laboratory methodology improvement. The aim of this study is to introduce standardised multicolour FC in the diagnosis of haematological malignancies using chronic lymphocytic leukaemia (CLL) as the proof of principle. In addition, we aim to document the incidence of CLL from the year 2011 to 2016 in the Tygerberg Academic Hospital (TAH) catchment area of Cape Town, South Africa (SA).

Methods: Twenty CLL patients were recruited at TAH. Bio-specimens were prepared and analysed on the Beckman Coulter Navios flow cytometer using Euroflow™ standardised FC protocols and immunophenotypic panels with two tubes for detecting B-cell chronic lymphoproliferative disorders (B-CLPD). Tube 1 included CD20, CD4, CD45, CD8, Ig-Kappa, CD56, Ig-Lambda, CD5, CD19, TCRyσ, CD3 and CD38. Tube 2 included CD20, CD45, CD23, CD10, CD79b, CD19, CD200 and CD43. Combined, the two tubes identified CLL from other B-CLPD. The CLL immunophenotypic profiles were stored in a database using the compass tool of the Infinicyt™ FC software. In addition, the clinical records of patients diagnosed with CLL at TAH over a 6-year period from the year 2011 to 2016 were retrieved and analysed using descriptive statistics.

Results: In comparison with the SA National Health Laboratory Service (NHLS) results at TAH, the Euroflow™ standardised multicolour FC panels and protocols are suitable for immunophenotyping CLL in this SA population. An immunophenotype database for 20 CLL diagnosed at TAH was constructed using the EuroFlow™ standardised multicolour FC panels. For the epidemiology part of the study, a total of 80 CLL patients were studied. There were slightly more females (51.2%) and the mean age at diagnosis was 67 years (37 to 95). Ninety one percent of the patients were aged 50 years and above. Males presented with the disease at a younger age (mean 63 years) than females (mean 70 years). CLL concurrent with HIV was not common (4%) and these patients were younger than 50 years. Twenty-one patients were tested for chromosomal aberrations trisomy 12 and deletion 11q, 24% and 33% were positive respectively. Deletion 13q was assessed in 25 patients and 16% were positive. Twenty
patients were tested for deletion 17p and all were negative. Translocations t(8;14), t(11;14) and t(14;18) were negative in 1, 8 and 4 patients respective.

**Discussion:** Accurate and consistent laboratory techniques and strict standardisation in FC enhances the confidence in inter-laboratory studies. Establishment of haematological malignancy immunophenotype databases would allow for faster differential diagnoses of new disease cases which is needed within our setting. Furthermore, these databases permit clear identification of atypical cases. Monitoring haematological malignancy trends is a crucial step in the management of the disease.
Opsomming

Agtergrond: Vloeisitometrie (FC) immunofenotipering is van kardiale belang in die diagnose en standaardisering van hematologiese kwaardighede/maligniteite. Die FC tegnieke vereis ten einde betroubare en herhaalbare resultate wat belangrik is in inter-laboratorium studies vir die verbetering van laboratorium metodiek. Die doel van hierdie studie was om gestandaardiseerde multi FC in die diagnose van hematologiese maligniteite te gebruik met chroniese limfositiese leukemie (CLL) as bewys van beginsel. Daarbenewens, streef ons daarna om die voorkoms van CLL te dokumenteer in die tydperk van 2011 tot 2016 in die Tygerberg Akademiese Hospitaaal (TAH) opvanggebied van Kaapstad, Suid-Afrika (SA).

Metodes: Twintig CLL pasiënte was gewerf by TAH. Bio-monsters is voorberei en ontleed op die Beckman Coulter Navios vloeisitometer met behulp van Euroflow™ gestandaardiseerde FC protokolle en immunofenotipe panele met twee buise, vir die opsporing van B-sel chroniese limfoproliferatiewe versteurings (B-CLPD). Buis 1 het CD20, CD4, CD45, CD8, LG-Kappa, CD56, LG-Lambda, CD5, CD19, TCRyσ, CD3 en CD38 bevat. Buis 2 het CD20, CD45, CD23, CD10, CD79b, CD19, CD200 and CD43 bevat. In kombinasie het die twee buise CLL van ander B-CLPD onderskei. Die CLL immunofenotipe profiele is gestoor in 'n databasis met behulp van die kompas instrument (“compas tool”) van die Infinicyt™ FC sagteware. Daarbenewens was die kliniese rekords van pasiënte gediagnoseer met CLL by TAH, oor 'n tydperk van 5 jaar vanaf die jaar 2011-2016, opgespoor en ontleed met behulp van beskrywende statistiek.

Resultate: In vergelyking met die SA Nasionale Gesondheids Laboratoriumdienste (National Haleth Laboratory Services - NHLS) resultate by TAH, is die Euroflow™ gestandaardiseerde multi FC paneel en protokolle geskik vir die immunofenotiperinig van CLL in die SA bevolking. 'n Immunofenotipe databasis vir 20 CLL gevalle, gediagnoseer by TAH, is gebou met behulp van die EuroFlow™ gestandaardiseerde multi FC paneel. Vir die epidemiologiese deel van hierdie studie is 'n totaal van 80 CLL pasiënte bestudeer. Daar was effens meer vroue (51,2%) en die gemiddelde ouderdom by diagnose was 67 jaar (37-95). Een en negentig persent van die pasiënte was 50 jaar en ouer. Mans het die siekte vertoon op 'n vroeër ouderdom (gemiddeld 63 jaar) as vroue (gemiddeld 70 jaar). CLL tesame met MIV was nie algemeen nie
(4%) en hierdie pasiënte was jonger as 50 jaar. Een en twintig pasiënte wat getoets was vir chromosoomafwyking, trisomie 12, en verwydering van 11q, was onderskeidelik 24% en 33% positief. Verwydering van 13q is getoets in 25 pasiënte en 16% was positief. Twintig pasiënte was getoets vir verwydering 17p en almal was negatief. Translokasies t (8; 14), t (11; 14) en t (14; 18) was negatief in 1, 8 en 4 pasiënte, onderskeidelik.

**Bespreking:** Akkurate, konsekwente laboratoriumtegnieke en streng standaardisering in FC verhoog die vertroue in inter-laboratorium studies. Die vestiging van immunofenotipe databasisse vir hematologiese maligniteite sal lei tot vinniger differensiële diagnose van nuwe siekte gevalle wat nodig is in ons omgewing. Verder sal hierdie databasisse die duidelike identifisering van atipiese gevalle toelaat. Die monitering van hematologiese maligniteit tendense is 'n belangrike stap in die bestuur van die siektes.
Dedication

For my parents

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List of Figures

**Figure 1.1:** Diagram illustrating light chain and heavy chain rearrangement

**Figure 1.2:** Schematic illustration of B-cell differentiation and relationship to B-cell neoplasms

**Figure 1.3:** Morphology of CLL

**Figure 1.4:** Schematic illustration of flow cytometry interrogation of stained sample

**Figure 1.5:** Analysis of peripheral blood for chronic lymphocytic leukaemia flow cytometry data using flow cytometry analysing software

**Figure 1.6:** Diagnostic flow diagram showing potential usage of EuroFlow markers

**Figure 2.1:** Workflow for sample collection, routine diagnosis, EuroFlow flow cytometry and immunophenotype database build up using Infinicyt™ software

**Figure 2.2:** Lymphocytes gated (red) according to Euroflow SOP.

**Figure 2.3:** Cytometer control window and parameters selection on the Navios flow cytometry software

**Figure 2.4:** Gating Singlet beads-population P1 and 7th Peak

**Figure 2.5:** EuroFlow and NHLS gating strategies, including used antibody markers.

**Figure 2.6:** Representative illustration of lymphocyte gating

**Figure 2.7:** Representative illustration of B-cells gating from the lymphocytes.

**Figure 2.8:** Workflow for data collection, CLL diagnosis confirmation and grouping of data according to gender, age, HIV, and cytogenetic properties

**Figure 3.1:** Representative diagram from one of the analysed samples illustrating the typical CLL immunophenotype profile using the EuroFlow flow cytometry panels.

**Figure 3.2:** Representative diagrams from one of the analysed sample illustrating the typical CLL immunophenotype using the NHLS flow cytometry panels.

**Figure 3.3:** Representation of population parameters for patients added to the Tygerberg Academic Hospital CLL database
Figure 3.4: Diagrams represent CLL immunophenotype database at Tygerberg Academic Hospital.

Figure 3.5: CLL incidence distribution was erratic, changing yearly with no defined pattern.

Figure 3.6: CLL distribution by age at Tygerberg Academic Hospital.

Figure 3.7: Yearly average age of men and women at diagnosis of CLL at Tygerberg Academic Hospital.

Figure 3.8: Distribution of age at diagnosis of CLL at Tygerberg Academic Hospital from 2011 to 2016

Figure 3.9: Representation of distribution of CLL by HIV infection at Tygerberg Academic Hospital

Figure 3.10: Representative bar chart illustrating cytogenetic characteristics of CLL patients at Tygerberg Academic Hospital

Figure 4.1: Typical flow cytometry experiment with cluster to show areas of common variables.

Figure 4.2: Variability between the same antibody type with different clones
List of Tables

Table 1.1: Required and recommended markers for use in the diagnosis of CLL with reagent specification based on expression patterns in normal peripheral blood.

Table 1.2: LST markers used for identification of lymphoid cells in patients with CLPD

Table 1.3: EuroFlow B-CLPD panel

Table 1.4: Limited EuroFlow BCLPD antibody panel for B-cell chronic lymphocytic leukaemia

Table 2.1: List of antibody volume per test, manufacturer, and clone.

Table 2.2: Target MFI values for the 7th peak (Trueview Settings Set 1) for the Navios flow cytometer instrument.

Table 2.3: Antibody panel used for compensation

Table 2.4: EuroFlow limited B-CLPD panel including LST panel

Table 3.1: Demographic data of participating CLL patients

Table 3.2: EuroFlow limited B-CLPD and NHLS flow cytometry panels for CLL

Table 3.3: Cost analysis of NHLS and EuroFlow™ flow cytometry panels for the diagnosis of CLL

Table 3.4: Characteristics of TAH patients with newly diagnosed CLL from 2011 to 2016

Table 3.5: Prognostic cytogenetics profile of patients diagnosed with CLL at TAH from 2011 to 2016

Table 4.1: Positive and negative reference lymphocyte populations used for the various markers and their gating strategy for the EuroFlow™ LST
# Table of Contents

Declaration .............................................................................................................................. i

Abstract .................................................................................................................................. ii

Opsomming .............................................................................................................................. iv

Dedication ............................................................................................................................. vi

Acknowledgements ............................................................................................................... vi

List of Figures ....................................................................................................................... vii

List of Tables ......................................................................................................................... ix

CHAPTER 1: LITERATURE REVIEW ................................................................................... 1

1.1 Introduction .................................................................................................................. 1

1.1 Lymphocytes and B-cells lymphopoiesis ...................................................................... 1

1.1.1 Primary Phase of B-cells development .................................................................. 2

1.1.2 Secondary phase of B-cells development .............................................................. 4

1.2 Chronic lymphocytic leukaemia .................................................................................... 6

1.3 Pathophysiology of chronic lymphocytic leukaemia ...................................................... 7

1.4. Diagnosis of chronic lymphocytic leukaemia .............................................................. 8

1.4.1 Morphology............................................................................................................ 8

1.4.2 Cytogenetic features in chronic lymphocytic leukaemia ......................................... 9

1.4.3 Other chromosomal abnormalities ........................................................................ 13

1.4.4. Next generation sequencing ................................................................................ 14

1.4.5. Flow cytometry .................................................................................................... 16

1.6 EuroFlow flow cytometry diagnostic method ............................................................... 19

1.6.1 EuroFlow Limited BCLPD panel ........................................................................... 23

1.7 Present study ..............................................................................................................24

CHAPTER 2: MATERIALS AND METHODS ........................................................................26

2.1 Study cohort ................................................................................................................26

2.1.1 Inclusion Criteria ................................................................................................... 27

2.1.2 Exclusion Criteria ................................................................................................. 27
2.1.3 Sample collection ...................................................................................................27

2.2 Flow cytometry immunophenotyping ........................................................................28
2.2.1 Daily quality checks of the Navios flow cytometer ...............................................28
2.2.2 EuroFlow standardised laboratory procedure .......................................................29

2.3 Detailed Instrument Set up Procedure .......................................................................29
2.3.1 Setting up forward scatter (FS) and Side scatter (SS) parameters (Navios Acquisition software) .....................................................................................................29
2.3.2 Setting up of PMT voltages for target fluorescence channels (Navios Acquisition software) .................................................................................................................30
2.3.3 Fluorescence Compensation Settings ..................................................................33
2.3.4 Flow cytometry Immunophenotyping ..................................................................35

2.4 Sample preparation and staining for flow cytometric data acquisition ....................35
2.4.1 Staining of back bone markers – Tube 2 only .......................................................37
2.4.2 Staining of surface markers ..................................................................................37

2.5 Gating Strategy ...........................................................................................................38
2.5.1 Infinicyt™ software .............................................................................................42

2.6 Incidence of chronic lymphocytic leukaemia at Tygerberg Academic Hospital over a 6-year period, 2011 to 2016 .................................................................43
2.6.1 Statistical analysis .................................................................................................44

CHAPTER 3: RESULTS ......................................................................................................45
3.1 Standardised multicolour flow cytometry study ............................................................45
3.1.1 Population description of CLL participants ............................................................45
3.1.2 Flow cytometry analysis .........................................................................................46
3.1.3 Antibody panels and analysis gating strategies ......................................................47
3.1.4 Establishment of CLL immunophenotype database ..............................................51
3.2 Incidence of CLL within the Tygerberg Academic Hospital Service Area .................55

CHAPTER 4: DISCUSSION ...............................................................................................63
4.1 Multicolour flow cytometry .........................................................................................64
4.1.1 Protocol, marker panel and gating strategies comparisons between EuroFlow and NHLS flow cytometry CLL based assays .........................................................64
4.1.3 Establishment of Immunophenotypic database using Infinicyt™ flow cytometry software ........................................................................................................................70
4.1.4 Cost Analysis comparison between NHLS vs EuroFlow panels .................................................................71
4.1.4 Limitations of the multicolour flow cytometry study .............................................................................71
4.2 CLL Incidence study .....................................................................................................................................76
  4.2.1 Six-year CLL incidence at TAH .............................................................................77
  4.2.2 Limitations of Incidence study ...............................................................................80
  4.2.3 Recommendations ................................................................................................ 80
4.3. Conclusions ............................................................................................................81
Reference ........................................................................................................................................................82
CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Haematological malignancies are cancers of the lymphatic system, blood and the bone marrow and are commonly referred to as lymphomas and leukaemias. Early and accurate diagnosis of these haematological malignancies is important to the overall survival of the patient. In their advanced stages, both lymphomas and leukaemias are known to involve the bone marrow.

Lymphomas and leukaemias are malignancies of leukocytes (white blood cells) which are cells of the immune system that are responsible for getting rid of foreign particles (antigens) that may invade the body. Different types of white blood cells exist, and these include neutrophils, eosinophils, basophils, monocytes, and lymphocytes. The different types of white blood cells have different modes of action and are specific to different kinds of invading antigen.

Of interest in this review are the lymphocytes commonly known as B-cells and their malignancies, with emphasis on the diagnosis of chronic lymphocytic leukaemia (CLL) using flow cytometry. Thus, for this purpose a condensed and simplified overview on these key areas are covered to provide context to subsequent discussions.

1.1 Lymphocytes and B-cells lymphopoiesis

During the lymphocyte development process, self-renewing hematopoietic stem cells develop into common lymphoid progenitors (CLP) (Kondo 2010). The CLP is committed to the development of the lymphoid lineage which results in the production of T-cells and B-cells.

T-cells are important in adaptive immunity, where they act by directly destroying autologous and infected cells, for example cells infected by viruses and cancerous cells. On the other hand, B-cells are important in humoral immunity where they act differently by producing antigen specific antibodies that eliminate foreign and
autologous threats (Cooper & Alder 2006; Zhang & Kipps 2014). For the purpose of
this review and subsequent research study, only B-cells will be discussed further.

B-cells derive their name from the bursa of the Fabricius, a lymphoid organ of birds
and it is where B-cells were first identified (Glick et al. 1956; Cooper et al. 1966). In the
human body, B-cells develop in the bone marrow. The B-cell development process
occurs in two phases known as the primary and secondary phases. The two phases
are controlled by a series of signals generated on the surface of the B-cells due to
cellular interactions and stimulation by various growth factors (Lenz & Staudt 2010).

Different maturation stages of B-cells are characterised by the expression of different
cell surface markers and Immunoglobulin (Ig) gene rearrangement (Ademokun et al.
2010). Different B-cell neoplasms arise at different maturational stages of normal B-
cells (Swerdlow et al. 2008).

1.1.1 Primary Phase of B-cells development

During the human foetal development, B-cells originate and differentiate in the liver.
After birth, the B-cell development shifts to the bone marrow where stromal cells
support their differentiation into mature B-cells (Abbas et al. 2014).

Stromal cells in the bone marrow supply differentiation promoting growth factors,
chemokines, and cytokines that influence the production of B-cells from CLPs.
(Ademokun et al. 2010). The earliest identifiable cells in the B-cell lineage development
are known as pro-B-cells. Classification of the different stages of B-cells development
is done based on the presence of specific surface cell receptors and Ig rearrangements
(Hardy et al. 2000).

In the pro-B-cells stage of development, the B-cells are identifiable by surface
receptors CD34⁺, CD10⁺, CD19⁺ (Sanz et al. 2010) and CD43 (Abbas et al. 2014).
The first rearrangement of Ig genes is initiated at heavy chain region. Somatic
recombination involving the Diverse (D) and Joining (J) gene sections leads to the
formation of the D-J sequence and deletion of DNA. Thereafter, the Variable (V) gene
is added resulting in the VDJ exon sequence (Figure 1.1). Transcription of the
recombined heavy chain gene follows to produce μRNA which is subsequently spliced
to from mRNA for heavy chain μ after the removal of introns. Success or failure of this
process determines B-cells development progression to the next stage. If successful, the produced RNA will be translated to μ protein. Only about half of the pro-B-cells produce and express the μ heavy chain to become pre-B-cells (Melchers et al. 2000; Abbas et al. 2014).

Pre-B-cells carry the pre-B-cell receptor known as pre-BCR which develops from the rearranged heavy chains and consists of a heavy chain and surrogate light chains. The pre-B-cells are known to express the surface markers CD10, CD19, CD20 and HLA-DR (Swerdlow et al. 2008). The pre-B-cells proliferate and leave the primary phase of development as naïve B-cells and they enter the secondary phase of development which occurs in the secondary lymphoid organs. Before leaving the bone marrow, the cells are vigorously tested for auto reactivity on their antigen receptors (Kyewski & Klein 2006). The naïve B-cells are not capable of responding to antigen invasion, however, it has been reported that they can be activated in special cases such as inflammation (Pieper et al. 2013).
1.1.2 Secondary phase of B-cells development

This stage of development occurs in the spleen, a secondary lymphoid organ where the naïve B-cells undergo maturation and selection. Activation of B-cells is either T cell independent or dependent. In T-cell independent activation, antigens stimulate B-cells in the absence of helper T-cells (CD4+). These antigens exist as protein free structures, for example nucleic acids and polysaccharides. The antigens involved in this process are classified as thymus-independent type 1 (TI 1) and type 2 (TI 2) antigens. The two antigens have different activation results with TI 1 antigens giving rise to memory B-cells production whilst TI 2 does not (Vinuesa & Chang 2013).

The T-cell dependent activation pathway is stimulated by the actions of antigen specific T-cells and follicular dendritic cells (Lenz & Staudt 2010). Follicular dendritic cells secrete a chemokine known as CXCL13 that is important in downstream CD4+ interactions. This T-cell independent pathway relies on the activity of the CD4+ T-cells to activate B-cells. The pathway involves a dendritic cell and antigen complex which, in the presence of activation molecules such as CD40, CD80 and CD86 and cytokines such as interleukins 1 and 12 and tumour necrosis factor (TNF)-α results in the activation of naïve CD4+ T-cells. The activated CD4+ T-cells causes increase expression of the CD40 ligand and interact with naïve B-cells in the lymphoid region to activate transcription factors NF-κB and AP-1 that are active in the germinal centre reaction (Gerondakis & Siebenlist 2010).

The germinal centre is divided into the light zone and the dark zone based on their appearance under a microscope. These two zones have different roles. In the dark zone, B-cells called centroblasts rapidly proliferate, differentiate, and mutate their antibody encoding DNA through a process called somatic hypermutation.

Somatic hypermutation involves Ig variable region mutations which lead to the formation of B-cells with increased or decreased affinity for a specific antigen (Ag) (Phan et al. 2006). Class switch recombination causes changes in the IgH class from IgM to IgG/IgA/IgE (Lenz & Staudt 2010). These DNA modifications are required for normal immune responses; however, in some cases they may give rise to DNA damage that may be pathologic in lymphomas and leukaemias (Swerdlow et al. 2008).
In the light zone, the B-cells undergo a selection process whereby they either survive or are destroyed based on their Ig receptor affinity for specific antigens. Only the B-cells with high affinity receptors survive and move on to the maturation stage whilst those with weak affinity receptors are marked for destruction (Peterson et al. 1992).

Along the different stages of B-cell development, abnormal processes can give rise to different B-cells malignancies depending on the stage of development (Figure 1.2). B-cells can be identified by their expression of primary B-cell markers that are known as pan B-cell markers. These surface expressions are CD19 and CD20. The different stages of B-cells development affected by different cancers are identified by immunophenotypic panels of antibodies that bind to specific surface expressions of deformed B-cells.
Figure 1.2: Schematic illustration of B-cell differentiation and relationship to B-cell neoplasms. DLBCL, diffuse large B-cell lymphoma; CLL/SLL, chronic lymphocytic leukaemia/small lymphocytic lymphoma; MALT, mucosa-associated lymphoid tissue; AG, antigen; FDC, follicular dendritic cell. Red bar is Ig heavy chain gene (IGH) rearrangement; blue bar is Ig light chain gene (IGL) rearrangement; black insertions in the red and blue bars indicate somatic hypermutation. Extracted from Swerdlow et al. 2008.

1.2 Chronic lymphocytic leukaemia

The World Health Organisation (WHO) defines chronic lymphocytic leukaemia/small lymphocytic lymphoma (CLL/SLL) as a post germinal centre (GC) neoplasm made up of monomorphic, round to slightly irregular B-lymphocytes in the peripheral blood (PB), bone marrow (BM), spleen and lymph nodes, admixed with pro-lymphocytes and paraimmunoblasts forming proliferation centres in tissue infiltrates (Swerdlow et al. 2008). SLL is the tissue equivalent of CLL and the WHO identifies the two malignancies
as one entity with different manifestations, thus the diagnosis and treatment are the same (Swerdlow et al. 2008). For this review, the term CLL will be used henceforth in future chapters and discussions.

CLL is the most common haematological malignancy in the western countries with approximately 22 000 new cases expected in the United States of America (USA) for the year 2017 (American Cancer Society 2017). Despite the high rates reported in the Western countries, in South Africa, studies focusing on CLL occurrence are scarce. A previous study by Nel et al. 1998, showed that CLL incidence patterns at the Universitas and Pelonomi Hospitals in Bloemfontein were similar to incidence patterns reported world-wide. CLL has an estimated incidence rate that rises from between < 1 and 5.5 per 100,000 and the median age of 64–70 years at diagnosis (Redaelli et al. 2004). Interestingly, CLL is increasingly becoming diagnosed in younger adults (Parikh et al. 2013) and is more common in males with a predominance of 1.5 (Cantú et al. 2013). Previous studies have noted familial aggregation as a trait of CLL (Goldin et al. 2009).

1.3 Pathophysiology of chronic lymphocytic leukaemia

Most CLL patients are asymptomatic and some patients may present with incidentally discovered lymphocytosis in the peripheral blood for the duration of at least 3 months (Hallek 2015). Others present with tumour burden signs and symptoms such as fatigue, infection, splenomegaly, hepatomegaly, extra-nodal infiltrates organomegaly, autoimmune haemolytic anaemia and/or lymphadenopathy (Hsi 2012; Swerdlow et al. 2008).

CLL is a clonal disease of B-cells that have deregulated programmed cell death (apoptosis) (Guarini et al. 2003; Azadi et al. 2014). Clonogenic events give rise to mature-appearing lymphocytes which may carry proliferative and/or survival advantage over their normal counterparts (Guarini et al. 2003). CLL cells survive for an abnormally long time due to deregulated apoptosis in their cell cycle and CLL is characterised by a generally incurable clinical course (Scarfò et al. 2016). Progressive accumulation of clonal cells and their products may cause peripheral blood lymphocytosis (5,000 B-cells/uL) and bone marrow infiltrates and may lead to the enlargement of tissues (e.g. lymphadenopathy, splenomegaly) (Guarini et al. 2003).
Chronic lymphocytic leukaemia is characterised by the co-expression of surface markers CD5, CD19 and CD23 as well as low levels of surface Immunoglobulin by B-cells in blood, bone marrow and secondary lymphoid tissues (Swerdlow et al. 2008; Zhang & Kipps 2014). The disease is clinically heterogeneous and follows either an indolent or aggressive course (Cheson 2001). Indolent CLL has a higher survival rate and is generally not treated until there is evidence of progression (Cheson 2001). The waiting period until treatment of indolent CLL is termed ‘watchful waiting’. In contrast, ‘watchful waiting’ is not practiced for aggressive CLL which needs prompt treatment to increase the chances of patient survival (Hallek et al. 2008).

1.4. Diagnosis of chronic lymphocytic leukaemia

Diagnostic processes are initiated due to clinical manifestations and laboratory findings (Davis et al. 1997; Barrena et al. 2011). Diagnosis establishment, prognostic classification, and treatment effectiveness evaluation are the three major applications for laboratory diagnostics in haematological malignancies (Swerdlow et al. 2008).

Diagnosis of CLL is made after persistent lymphocytosis of greater than 5000 B-cells/uL of mature lymphocytes in the peripheral blood in the absence of disease related symptoms, or tissue involvement other than bone marrow (Inamdar & Bueso-Ramos 2007) (Hallek et al. 2008)

Identification and characterisation of abnormal lymphocytes from normal and reactive cells are crucial steps to final diagnosis (van Dongen & Orfao 2012). Aberrant lymphocytes can be clearly distinguished from normal and reactive lymphocytes by morphology and flow cytometry (Matutes & Polliack 2000; Swerdlow et al. 2008; Barrena et al. 2011).

1.4.1 Morphology

Examination of the peripheral blood is usually the first step in the pathologic evaluation of suspected CLL cells (Hsi 2012). Smudge cells or Gumprecht nuclear shadows which are ruptured CLL cells, are commonly noticed in peripheral blood smears due to the fragility of the cell membrane (Matutes & Polliack 2000; Inamdar & Bueso-Ramos 2007) (Hallek et al. 2008).
In bone marrow and peripheral blood smears, CLL cells are seen as small lymphocytes with clumped chromatin and scanty cytoplasm (Inamdar & Bueso-Ramos 2007; Swerdlow et al. 2008) (Scarfò et al. 2016) (Figure 1.3). Lymph nodes, spleen and liver may also be infiltrated as well as other extra-nodal sites (occasionally involved) (Swerdlow et al. 2008).

In about 15% of CLL patients, increased numbers of prolymphocytes (PL) (>10%) may be noticed and this is termed CLL/PL (Inamdar & Bueso-Ramos 2007). When compared to typical CLL cells, prolymphocytes (Figure 1.3) are larger with less condensed nuclei and a single distinguished nucleolus (Kipps et al. 2017). Increased numbers of prolymphocytes have been linked to poor prognosis and increased resistance to treatment (Oscier et al. 2016).

**Figure 1.3: Morphology of typical CLL (A) with small cells carrying round nuclei and condensed chromatin and Mixed type or atypical CLL (B) represented by cells with nuclear irregularities and heterogeneity of size with occasional prolymphocyte containing a visible nucleolus. Extracted from Hsi 2012.**

### 1.4.2 Cytogenetic features in chronic lymphocytic leukaemia

Genetic features are important in the classification and diagnosis of lymphoid malignancies (Swerdlow et al. 2008). Studies in molecular genetics have greatly improved understanding of haematological malignancy diversity resulting in the discovery of oncogenic pathways as well as novel therapeutic targets (Sehn, H & Gascoyne, D 2015).
Characteristic genetic abnormalities exist for several lymphoid malignancies. These abnormalities are important in determining the biological features of different malignancies and can therefore be used for differential diagnosis (Swerdlow et al. 2008). Distinguishing between different kinds of lymphoid malignancies is critical because their management may be different (Dave et al. 2006). In addition, molecular features of tumours may also be used to predict the survival of patients after therapy (Shaffer et al. 2002).

Cytogenetic tests may be performed in the diagnosis of CLL. These tests are not essential for CLL diagnosis, however they give a clearer diagnostic picture and are recommended to predict the disease progression rate (Hallek et al. 2008). Polymerase chain reaction (PCR) DNA-based molecular assays, fluorescence in situ hybridization (FISH), conventional karyotyping are valuable laboratory techniques employed in determining chromosomal abnormalities in different malignancies (Swerdlow et al. 2008).

In karyotyping, culturing of lymphocytes is done using mitogens which stimulate them into active mitosis. The cells are harvested in the metaphase stage before being fixed and spread onto a microscopic slide where they are stained, usually by Giema stain for G-banding and R banding. This results in microscopically visible chromosome bands which are then photographed and arranged into pairs during assessment. The chromosomes are assessed for deletion and translocation abnormalities. Unlike FISH, karyotyping allows for the visualisation of the entire genome, individual cells, and chromosomes. The downside to this technique is the requirement of continuously growing cells and limited resolution of 5Mb. On the other hand, FISH has a resolution of 100kb-1Mb which is superior. In FISH, locus specific DNA labelled with fluorescent probes hybridises with patient DNA within the nucleus thus allowing the microscopic detection of abnormal chromosomes based on the fluorescent signals. FISH confers the advantage of shorter turnaround time than karyotyping because it does not rely on cell culturing which takes long. In contrast to karyotyping, the disadvantage of FISH is; it is a loci specific method which does not allow whole entire genome visualisation. However, FISH can be used to investigate almost any DNA; it can also be used for cells regardless of cell cycle stage and its results are for specific cells.
Cytogenetic features are crucial for the definition of different CLL subsets, whilst genetic changes within neoplastic cell populations are used to determine the prognosis of CLL patients (Döhner et al. 2000; D.-M. Wang et al. 2011). Some of the most common structural abnormalities of chromosomes in CLL are del17p, del11q, del13q, and Trisomy 12 (Kipps et al. 2017; Döhner et al. 2000). At least one of the aforementioned chromosomal alterations are carried in about 80% of CLL patients (Kipps et al. 2017). Even in the presence of these advances, CLL prognostic prediction has not reached a state where it can be completely reliable (L. Wang et al. 2011).

1.4.2.1 Deletions of the short arm of chromosome 17 (del 17p)

Five to eight percent of CLL patients unexposed to chemotherapy have deletions of the short arm of chromosome 17 (17p) (Hallek 2015). These deletions target the tumour suppressor gene TP53 that is located at the 17p13 locus (Garff-Tavernier Le et al. 2011). The deletion of tumour suppressor gene TP53 leads to the deregulation of numerous genes including microRNAs (miR-34a). TP53 deletions occur in 4-37% of CLL patients and are associated with poor prognosis (Zenz et al. 2010). TP53 mutations have also been associated with lower chemotherapy response and overall survival rates despite the presence or absence of del 17p (Seiffert et al. 2012).

Patients with the del 17p have rapidly progressing CLL disease that requires treatment after short periods of time and the patients have a short overall survival rate. Patients with the del 17p have marked resistance against genotoxic chemotherapies that cannot be overcome by the inclusion of anti-CD20 antibodies (Hallek 2015).

1.4.2.2. Deletions of the long arm on chromosome 11 (del 11q)

These deletions are predictors of poor prognosis (Hartmut Döhner et al. 1997) and are encountered in about 25% of advanced stage CLL cases and in about 10% of early stage CLL patients (Zenz et al. 2010). The 11q deleted region is in the miR-34b/c cluster (M. Fabbri et al. 2011). The deletions target the ATM gene which encodes for the proximal DNA damage response kinase ATM (Hallek 2015). Patients with the chromosome 11 (del 11q) deletion usually present clinically with bulky
lymphadenopathy, rapid progression, and a lower overall survival rate (Hartmut Döhner et al. 1997).

1.4.2.3 Trisomy 12

An additional chromosome 12 is relatively frequent, occurring in 10-20% of CLL patients (Puiggros et al. 2014). Information on the trisomy 12 carrying genes in the pathogenesis of CLL is relatively scarce (Hallek 2015). Previous studies identified potential genes that can be upregulated via a gene dosage effect and these include CDK4, P27, MYF6, HIP1R and MDM2 (Winkler et al. 2005) and more recently, association between trisomy 12 and NOTCH1 mutation has been reported (Balatti et al. 2012). MDM2 is a ligase that mediates the degradation of p53 and suggestions have been made that increasing its expression levels results in the deregulation of the cell cycle (Puiggros et al. 2014). MDM2 overexpression is thought to significantly reduce the p53 effector microRNA, miR-34a, with the consequence of aggressive CLL and reduced overall survival. Trisomy 12 imparts an intermediate prognosis (Döhner et al. 2000), however, its prognostic relevance is still debatable (Seiffert et al. 2012). The abnormality has been linked to high levels of CD38 antibody marker expression and is commonly noticed in atypical CLL (Puiggros et al. 2014).

1.4.2.4 Deletion of the long arm of chromosome 13 (del 13q)

This is the most common abnormality in CLL with approximately 55% of patients carrying this trait (Hallek 2015). The prognosis for this group of patients is good because they have indolent CLL (Döhner et al. 2000). Their target gene is a chromosome segment coding for miR-15a and miR-16-1 which are located in the critical chromosomal region of del 13q14 (Calin et al. 2008).

The miR-15a/16-1 microRNA cluster functions as a tumour suppressor targeting the oncogene BCL2 (Pekarsky & Croce 2014). Loss of miR-15a/16-1 in CLL patients with the 13q deletion results in high levels of BCL2 and P53 (Moreno & Montserrat 2010). BCL2 is an oncogene that inhibits cell death and promotes survival (Cimmino et al.
High levels of BCL2 result in a reduction in the number of apoptotic cells and high levels of p53 suppress the tumour burden and keeps it relatively low, making the CLL course indolent (Balatti et al. 2016).

1.4.3 Other chromosomal abnormalities

1.4.3.1 Deletion of 6q (del6q)

Deletions of the long arm of chromosome 6 are common in malignancies of the lymphoid system (Döhner & Stilgenbauer 1999). However, a study by Cuneo et al., 2004 observed that only a minority of CLL cases carrying del 6q as an early chromosome defect defining the stem line; Cuneo et al. 2004 suggested that 6q chromosome deletion may help in the identification of a distinct cytogenetic entity of CLL. A more recent study reported that del 6q is common in patients with progressive disease and suggested FISH probes detecting 6q for clinical practice (Wang et al. 2011). In addition, Wang et al. 2011, went on to suggest del 6q,23 as a marker of intermediate prognosis between CLL cases with del(11q22.3) or del(17p13) and those cases with del(13q14).

1.4.3.2 Translocation of 14q32

In CLL, structural aberration on chromosome 14 commonly involve the genes for the heavy chains on band q32 on the long arm (Döhner & Stilgenbauer 1999). The q32 band is normally involved in the gene rearrangements during normal B-cells development (Juliusson & Gahrton 1993). 14q32 translocation has been reported in approximately 7% of CLL patients and the abnormality represents an adverse prognosis (Cavazzini et al. 2008).

1.4.3.3 Translocations

Translocation t(8;14) is a confirmatory abnormality for Burkitt’s lymphoma (Burmeister et al. 2013). In mantle cell lymphoma (MCL), t(11;14) is common (Vorobyev et al. 2011) whereas t(14;18) is frequent in follicular lymphoma (FL) (Leich et al. 2009; Horsman et al. 1995). These translocations are important because they represent malignancies
that are heterogeneous with CLL. The cytogenetic properties are usually assessed for in suspected cases of atypical CLL which may be difficult to distinguish from the 3 lymphomas using morphology and flow cytometry.

1.4.3.4 Ig Heavy Chain Variable Gene (IGVH) Mutation Status

Immunoglobulin mutational status is useful for predicting the clinical course in individual malignancy cases. \textit{IGVH} provides prognostic classification as well as tumour burden assessment (Hallek et al. 2008; Goede et al. 2014). The mutational status is dependent on the variable region of the \textit{IGVH} (Call et al. 2014).

Unique Ig rearrangements of VDJ genes located on chromosome 14 occur during B-cell development to form a distinct DNA sequence coding for the individual heavy chain (\textit{V}_{\text{H}}) of a given B-cell clone (Swerdlow et al. 2008). Previous studies have demonstrated that unmutated \textit{IGVH} is associated with progressive disease and poor prognosis while somatic hypermutations of the \textit{IGVH} are associated with stable disease and higher survival rates (Ritgen et al. 2003; Balatti et al. 2015).

1.4.4. Next generation sequencing

Large scale sequencing approaches led to the realisation that CLL has high genetic and epigenetic heterogeneity (Scarfò et al. 2016). Previous studies on \textit{TP53} mutated sub-clones in CLL reported that small tumour cell populations of low clonal abundance are capable of significantly influencing the disease course (Rossi et al. 2014). Other genes associated with CLL include NOTCH signalling (NOTCH1), mRNA processing (SF3B1) and DNA damage and cell cycle control (BIRC3) and these have been associated with poor prognosis. (Foà et al. 2013). Inflammatory pathway mutations involving MYD88 have also been identified (Gruber & Wu 2014). The four mentioned alterations have been shown to be significant determiners of prognosis in large cohort studies and proposals to incorporate them in prognostic nomograms have been made. (Scarfò et al. 2016).
1.4.4.1 TP53

Routine assessment of TP53 mutation or deletion is done in the diagnostic workup for CLL. This mutation is low at diagnosis and is found in 5-15% of CLL cases (Robles & Harris 2010; Scarfò et al. 2016). TP53 mutation or deletion has poor free survival rates, therapy response and overall survival of patients with CLL (Döhner et al. 2000). TP53 mutations are common in unmutated IGVH genes cases (Zenz et al. 2010). The mutations found in the remaining allele of 80-90% of patients carrying the del17p results in functional inactivation of the p53 pathway (Scarfò et al. 2016).

1.4.4.2 NOTCH1

NOTCH signalling controls Nuclear Factor kB (NFkB) which regulates the expression of several antiapoptotic molecules, including the c-IAP proteins (Braeuer et al. 2006). The signalling pathway is involved in CLL cell survival (Rosati et al. 2009). NOTCH1 mutations have been reported to occur in about 10% of CLL cases at diagnosis and are more commonly seen in advanced stages of the disease (G. Fabbri et al. 2011).

1.4.4.3 SF3B1

This mutation is fairly uncommon at diagnosis and is found in 5-7% of new patients (Scarfò et al. 2016b; Wang et al. 2011). Mutations in SF3B1 are common in del11q CLL case and are associated with poor prognosis (L. Wang et al. 2011).

1.4.4.4 BIRC3

The BIRC3 gene is associated with apoptosis inhibition and NFkB mutations are mutually exclusive with TP53 (Scarfò et al. 2016). This gene is located near the ATM gene at 11q22. BIRC3 gene mutations are uncommon and account for approximately 4% of cases in treatment naive CLL. In contrast, abnormalities in the gene have been detected in approximately 24% of CLL cases that are refractory to fludarabine therapy. BIRC3 abnormalities have also been reported as relatively low in progressive patients that are sensitive to fludarabine thereby suggesting the gene might be linked specifically with chemotherapy refractory CLL (Rossi et al. 2012).
1.4.5. Flow cytometry

Immunophenotyping is done via flow cytometry (Kalina et al. 2012). Flow cytometry is a laboratory analytical method that enables the measurement of antigen expression on a specific cell population (Njemini et al. 2014). The technique allows for the identification of cell characteristics as the individual cells suspended in a fluid at high pressure passes through a laser beam (Figure 1.3) (Brown & Wittwer 2000). The laser beam allows for each cell's physical properties to be measured hence determining cell size and its internal and/or surface complexity (Brown & Wittwer 2000; Maecker et al. 2012).

Identification of specific cell populations is done via the use of specific fluorescent probes. The fluorescent probes are attached to antibody markers of different cell populations. These antibodies subsequently attach to specific cytoplasmic and surface cell receptors during staining in sample preparation. The stained cells are then interrogated using a flow cytometer. As the cell-antibody-fluorescent probe complex passes through the laser beam in a flow cytometer, the complex absorbs light energy and gets excited to a higher energy state after which it must return to its resting phase.

As the complex returns to its resting energy state, it emits the light energy which is directed towards wavelength specific detectors by filters. The wavelength of this emitted light is dependent on the fluorescent probe. The detectors then relay the message to the Analog Digital Converter which allows the computer to visualise the message. In this manner, flow cytometry can give detailed information of specific cell characteristics that aid in cell population identification and enumeration (Figure 1.4).
Haematological malignancies are identified in flow cytometry using specific combinations of antibody markers. The antibody markers expression levels on targeted cells help to determine cell population characteristics, a technique known as immunophenotyping (Swerdlow et al. 2008). CLL cells are CD5, CD19 and CD23 co-expressing B-cells that exhibit clonality (Swerdlow et al. 2008; Hallek et al. 2008). Clonality of the cells is confirmed by their restricted expression of either kappa or lambda light chains on the B-cells surface membrane (Moreau et al. 1997). In comparison to normal B-cells, CLL cells express low CD20, low surface Ig and low to negative CD79b (Moreau et al. 1997; Hallek 2015) and are negative for FMC7 as shown in Figure 1.5 (Inamdar & Bueso-Ramos 2007).

Figure 1.4: Schematic illustration of flow cytometry interrogation of stained sample. Adapted from Brown & Wittwer 2000.
Figures 1.5: Analysis of peripheral blood for chronic lymphocytic leukaemia data using flow cytometry analysing software. Lymphocytes are gated using CD45-scattered analysis. The plot CD5 vs CD19 is used to demonstrate dual positive neoplastic lymphocytes with weak CD19 fluorescence intensity. The plot of CD23 vs FMC7 illustrates positive staining of the cells for CD23 but no staining for FMC7. The cells are then plotted for both CD19 vs kappa and lambda, showing kappa light chain clonality with a 15:1 ratio of kappa to lambda for the dual CD19+, CD5+ cells. Extracted from Inamdar & Bueso-Ramos 2007.

Occasionally it is difficult to differentiate CLL from other haematological malignancies using basic CLL flow cytometry antibody panels. In these cases, significant immunophenotypic overlapping occurs between different but closely related lymphomas. A common example is distinguishing CLL from mantle cell lymphoma (MCL) (Spacek et al. 2014). The two entities are different, but they share an almost common antigen expression flow cytometry panel. Therefore, to accurately distinguish these two entities from each other, an additional array of flow cytometry antibody
markers are employed. Examples of these antigen expression markers are CD200, CD79b, CD43, CD10, CD22, and others (Table 1.1).

Table 1.1: Required and recommended markers for use in the diagnosis of CLL with reagent specification based on expression patterns in normal peripheral blood. Adapted from Rawstron et al. 2015.

<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
<th>Antigen</th>
<th>Expression (%positive vs %control)</th>
<th>Positive control population in normal peripheral blood</th>
<th>Negative control population in normal peripheral blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Required</td>
<td>CD19</td>
<td>Positive (&gt;95%)</td>
<td>CD20+ B-cells</td>
<td>CD3+ T-cells</td>
</tr>
<tr>
<td></td>
<td>CD5</td>
<td>Positive (&gt;20%)</td>
<td>CD3+ T-cells</td>
<td>CD56+ NK-cells</td>
</tr>
<tr>
<td></td>
<td>CD23</td>
<td>Positive (&gt;20%)</td>
<td>Naïve B-cells</td>
<td>Memory B-cells</td>
</tr>
<tr>
<td></td>
<td>CD20</td>
<td>Weak</td>
<td>CD19+ B-cells</td>
<td>CD3+ T-cells</td>
</tr>
<tr>
<td></td>
<td>Igk &amp; Igλ</td>
<td>Weak and restricted</td>
<td>CD20+ B-cells</td>
<td>CD3+ T-cells</td>
</tr>
<tr>
<td>Recommended</td>
<td>CD43</td>
<td>Positive (&gt;20%)</td>
<td>CD3+ T-cells</td>
<td>CD20+ B-cells</td>
</tr>
<tr>
<td></td>
<td>CD79b</td>
<td>Weak</td>
<td>CD20+ B-cells</td>
<td>CD3+ T-cells</td>
</tr>
<tr>
<td></td>
<td>CD22</td>
<td>Weak</td>
<td>CD20+ B-cells</td>
<td>CD3+ T-cells</td>
</tr>
<tr>
<td></td>
<td>CD200</td>
<td>Positive (&gt;20%)</td>
<td>CD19+ B-cells</td>
<td>CD3+ T-cells</td>
</tr>
<tr>
<td></td>
<td>CD10</td>
<td>Negative (&lt;20%)</td>
<td>Granulocites</td>
<td>Memory B-cells</td>
</tr>
</tbody>
</table>

*consensus

**1.6 EuroFlow flow cytometry diagnostic method**

Flow cytometry is crucial in the diagnosis and monitoring of patients with haematological malignancies. However, concerns regarding cost effectiveness and laboratory based variation of procedures have been raised (Braylan et al. 1997). Variables in flow cytometry are common during sample handling, instrument setup, data analysis and reagents (Maecker et al. 2012).
There is a growing interest in the standardisation of human immunophenotyping of haematological malignancies (Maecker et al. 2012). In 2005, more than 20 laboratories came together and created the EuroFlow consortium, an independent scientific consortium established with the aim of innovating and standardising flow cytometric immunophenotyping to improve diagnostic patient care (van Dongen et al. 2012).

The EuroFlow consortium put together specific combinations of individual antibody markers for the identification of cell lineage, characterisation of maturation stage and aberrant phenotype in the diagnosis of haematological malignancies. These antibody markers were arranged into highly sensitive multicolour flow cytometry panels that are suitable for immunophenotyping in the differential diagnoses of haematological malignancies. In addition, the EuroFlow consortium also standardised laboratory protocols for immunophenotyping of haematological malignancies.

EuroFlow antibody panels were designed for comprehensive immunophenotypic classification and diagnosis of haematological malignancies based on experience and knowledge from previous malignancy studies (van Dongen et al. 2012). The panels were optimised in multiple evaluation rounds using large numbers of patient samples (van Dongen et al. 2012; Kalina et al. 2012). The EuroFlow consortium then came up with standard operating procedures (SOP) for instrument set up including calibration, sample preparation, immunostaining and data acquisition (Kalina et al. 2012).

Precise and reproducible differential diagnoses of B-CLPD subtypes are important in obtaining the correct diagnosis. This is critical for optimal patient care because modern treatment regimens are tailored for specific B-CLPD subtypes (van Dongen et al. 2012). Current immunophenotyping strategies face increasing difficulties such as heterogeneous and overlapping immunophenotypic profiles among distinct entities; selection of crucial markers and the deletion of those that have become redundant are imperative in the diagnosis of haematological malignancies (van Dongen & Orfao 2012).

The EuroFlow group developed an optimized non-redundant combination of immunophenotypic markers for the diagnosis of B-CLPD using Flow cytometry. This was done using WHO 2008 guidelines (Swerdlow et al. 2008) as a diagnostic gold standard (van Dongen & Orfao 2012).
The group designed and evaluated an 8-colour, 12 marker combinations of antibodies in a single tube, called the lymphoid screening tube (LST) (Table 1.2).

Table 1.2: LST markers used for identification of lymphoid cells in patients with CLPD. Adapted from van Dongen et al. 2012.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Main normal population identified*</th>
<th>Positive diagnosis</th>
<th>Population subsetting</th>
<th>diagnostic subclassification</th>
<th>Potential minimal disease value</th>
<th>Prognostic Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>Mature lymphocytes and B-cell precursors</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD19</td>
<td>B-cells, T-cells and NK-cells by exclusion</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>CD20</td>
<td>B-cells, T-cells and NK-cells by exclusion</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>smlgκ &amp; λ</td>
<td>SmIg+ B-cells</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>CD38</td>
<td>Plasma cells and B-cells precursors</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>SmCD3</td>
<td>B-cells, T-cells and NK-cells by exclusion</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>CD4</td>
<td>CD4+ T-cells</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>CD8</td>
<td>CD8hi T-cells &amp; CD8lo NK-cells</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>CD56</td>
<td>NK cells</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>TCRγσ</td>
<td>TCRγσ+ T-cells</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>CD5</td>
<td>T-cells</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
</tbody>
</table>

The combination of antibody markers in the LST are effective in the detection of normal and phenotypically aberrant populations of mature B-, T- and NK-cells in peripheral blood (PB), bone marrow (BM), lymph nodes (LN) and other types of body tissues and fluids (Kalina 2013; van Dongen & Orfao 2012). The LST was a result of a seven-round process of design-evaluation-redesign of successive LST versions (van Dongen & Orfao 2012). Each LST was evaluated in a large but variable number of normal, reactive, and neoplastic patient samples obtained at diagnosis and other disease time...
points in parallel to routine approaches in participating EuroFlow laboratories. The final LST version showed 100% concordance with routine gold standard flow cytometry diagnostic practice and is able to discriminate between normal and abnormal lymphoid cells with 99% sensitivity (van Dongen & Orfao 2012).

The LST guides the need for further immunophenotyping with appropriate antibody panels for characterisation and accurate diagnosis of lymphoid malignancies using the different EuroFlow panels for B-CLPD, T-CLPD and NK-CLPD (Figure 1.6).

![Figure 1.6: Diagnostic flow diagram showing potential usage of EuroFlow markers. Extracted from van Dongen et al. 2012.](https://scholar.sun.ac.za)

The EuroFlow strategy is centred upon the identification of target populations in different tubes through common backbone markers in the same antibody panel. Backbone markers are added to all the tubes in a chosen panel. In the EuroFlow B-CLPD panel, pan B-cells markers CD45, CD19 and CD20 are the backbone markers (Table 1.3). The rest of the markers are characterisation markers with the ability to
identify B-cells malignancies such as CLL, MCL, FL, hairy cell leukaemia (HCL), diffuse large B-Cell lymphoma (DLBC), marginal zone lymphoma (MZL) and other BCLPD (van Dongen et al. 2012).

Table 1.3: EuroFlow B-CLPD panel. Adapted from van Dongen et al. 2012.

<table>
<thead>
<tr>
<th>Tube</th>
<th>PacB and CD4</th>
<th>CD20 and CD4</th>
<th>FITC</th>
<th>CD8 and smlgλ</th>
<th>CD56 and smlgk</th>
<th>PerCP-Cy5.5</th>
<th>PE-Cy7</th>
<th>APC</th>
<th>APC-H7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CD20</td>
<td>CD20</td>
<td>CD45</td>
<td>CD45</td>
<td>CD45</td>
<td>CD19</td>
<td>CD19</td>
<td>CD200</td>
<td>CD38</td>
</tr>
<tr>
<td>2</td>
<td>CD20</td>
<td>CD23</td>
<td>CD25</td>
<td>CD23</td>
<td>CD10</td>
<td>CD79b</td>
<td>CD19</td>
<td>CD200</td>
<td>CD43</td>
</tr>
<tr>
<td>3</td>
<td>CD20</td>
<td>CD31</td>
<td>LAIR1</td>
<td>CD11c</td>
<td>CD19</td>
<td>CD19</td>
<td>CD19</td>
<td>CD43</td>
<td>CD81</td>
</tr>
<tr>
<td>4</td>
<td>CD20</td>
<td>CD103</td>
<td>CD95</td>
<td>CD22</td>
<td>CD19</td>
<td>CXCR5</td>
<td>CD19</td>
<td>CD20</td>
<td>CD27</td>
</tr>
<tr>
<td>5</td>
<td>CD20</td>
<td>CD62L</td>
<td>CD39</td>
<td>HLA-DR</td>
<td>CD19</td>
<td>CD19</td>
<td>CD19</td>
<td>CD27</td>
<td></td>
</tr>
</tbody>
</table>

FITC: Fluorescein isothiocyanate; PE: Phycoerythrin; Cy5: cyanin5; Cy7: Cyanin7; APC: Allophycocyanin; PerCP: Peridinin-chlorophyll-protein; H7: Hilite; PacO: Pacific orange; PacB: Pacific blue; λ=lambda, k=kappa, sm=surface membrane. Backbone markers highlighted in yellow; Tube 1 is the LST.

1.6.1 EuroFlow Limited BCLPD panel

To save time and cost in the diagnosis of CLL, EuroFlow designed the B-CLPD panel (Table 1.4) such that the first two tubes (tube 1 and 2) can be used to fully distinguish CLL from all other B-CLPD. Combined with LST, tube 2 contains the characterisation antibody markers that completely identify and differentiate the CLL immunophenotype.
Table 1.4: Limited EuroFlow BCLPD antibody panel for B-cell chronic lymphocytic leukaemia. Tube 1 is the LST. Adapted from van Dongen et al. 2012.

<table>
<thead>
<tr>
<th>Tube</th>
<th>PacB</th>
<th>PacO</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP-Cy5.5</th>
<th>PE-Cy7</th>
<th>APC</th>
<th>APC-H7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CD20 and CD4</td>
<td>CD45</td>
<td>CD8 and smlgλ</td>
<td>CD56 and smlgk</td>
<td>CD5</td>
<td>CD19 and TCRYδ</td>
<td>smCD3</td>
<td>CD38</td>
</tr>
<tr>
<td>2</td>
<td>CD20</td>
<td>CD45</td>
<td>CD23</td>
<td>CD10</td>
<td>CD79b</td>
<td>CD19</td>
<td>CD200</td>
<td>CD43</td>
</tr>
</tbody>
</table>

FitC: Fluorescin isothiocyanate; PE: Phycoerythrin; Cy5: cyanin5; Cy7: Cyanin7; APC: Allophycocyanin; PerCP: Peridinin-chlorophyll-protein; H7: Hilite; PacO: Pacific orange; PacB: Pacific blue; λ=lamda, k=kappa; sm=surface membrane; Backbone markers are highlighted in yellow

1.7 Present study

In the present study, we aimed to introduce standardised multicolour flow cytometry for the diagnosis of haematological malignancies in the largest tertiary hospital in the Western Cape Province of South Africa, Tygerberg Academic Hospital (TAH). TAH is the largest tertiary hospital in the WC and the second largest in SA (Western Cape Government 2017) and along with Groote Schuur hospital (GSH) serves the estimated 6 million population of the WC province. (Statistics South Africa 2016) The SANAS accredited diagnostic company; NHLS provides a comprehensive 24-hour service to TAH and some of the clinics and secondary hospitals in the WC region. This service has also been expanded to include Eerste River Hospital and more peripheral clinics and serves as one of the referral centres for samples from the Eastern Cape, particularly from Nelson Mandela Academic Hospital. Together both the Clinical Haematology unit within TAH and the NHLS pathologist aid in the diagnoses and management of CLL patients that comes from these demographical areas.

The objective of the study was to qualitatively compare EuroFlow standardised protocols and expanded multicolour immunophenotypic panels within the local population presenting at TAH. In addition, we aimed to create an immunophenotypic database for haematological malignancies using CLL as a pilot. A further objective was to document and describe CLL incidence trends at TAH in the past 6-year period between the years 2011 and 2016.
This study forms part of a larger study that is currently being conducted by the Tygerberg Lymphoma Study Group (TLSG). The TLSG is doing extensive work in HIV related malignancies and intends to use the results of this study as a base for implementing an immunophenotypic database for the different HIV related malignancies (HRL) at TAH.
CHAPTER 2: MATERIALS AND METHODS

2.1 Study cohort

The study design is a proof of principle study to determine the suitability of EuroFlow standardised diagnostic panel in the South African population and setup. This study was approved by the Western Cape Provincial Government Research Committee, and the Health Research Ethics Committee (HREC) of the Faculty of medicine and Health Sciences of the University of Stellenbosch (HREC: S15/10/234) (Addendum 3 and 4) and forms parts of a larger study being conducted by the Tygerberg Lymphoma Study Group (TLSG). Patients and controls were recruited from TAH. All participants signed an informed consent prior to their participation.

Peripheral blood was collected from 20 consenting CLL positive individuals in EDTA BD vacutainer tubes (BD Vacutainer, San Jose, CA). (Figure 2.1). All CLL/SLL confirmation diagnoses were made within the Haematology Clinic, X block, TAH along
with laboratory confirmation from the National Health Laboratory Service (NHLS) unit at TBH. No treatment was given and no follow up was conducted. In addition, blood samples were also collected from 3 CLL negative ‘healthy’ controls to set up the Euroflow standardised protocol on the Beckman Coulter Navios 10 colour flow cytometer instrument (Beckman Coulter, Inc, USA).

2.1.1 Inclusion Criteria

All samples were obtained from the Haematology clinic at TAH. Patients were 18 years or older. Patients diagnosed with CLL and diagnosis was confirmed by the NHLS laboratory test were recruited for the study. Patients had to be untreated for CLL.

2.1.2 Exclusion Criteria

No exclusion of patients was done based on gender, race, or religious belief. Patients who were treated with Rituximab were excluded from the study. This was done because Rituximab has been proven to alter immunophenotype (Maeshima et al. 2009; Foran et al. 2001).

2.1.3 Sample collection

Following consent from patients, a total of 4-6ml of peripheral blood was collected into EDTA BD Vacutainer® tubes (Becton Dickinson (BD) Biosciences) and immediately transferred to the Department of Pathology, Division of Haematology, Faculty of Medicine and Health Sciences (FMHS), Stellenbosch University (SU) at room temperature. The samples were prepared and analysed within 12 hours of collection at the Beckman and Coulter/Stellenbosch University flow cytometry research laboratory at TAH.

For each patient, a separate study identifier was assigned. Minimal patient information data accompanying the specimen was kept separate and secure and only accessed by the principle investigator (PI).
2.2 Flow cytometry immunophenotyping

Flow cytometry is a technique whereby the physical and chemical characteristics of particles (cells) suspended in fluid are analysed as they individually pass through a laser beam. Cell components are fluorescently labelled (with cell markers) before being passed through the laser. As they pass through the laser, the cells become excited due to increased energy levels thus causing them to emit light at higher and different wavelengths depending on their markers. An optical to electronic coupling system is used to record the emission of fluorescence by the cells. The fluorescence can be measured to determine the various properties of a single particle, for example cell granularity, size, internal complexity, and fluorescence intensity. A combination of the cell properties combined can be used in the characterisation and differentiation of various diseases.

2.2.1 Daily quality checks of the Navios flow cytometer

A three laser and 10 Colour Beckman Coulter Navios flow cytometer instrument (Beckman Coulter, Inc, USA) was used for to acquire results for the study. Daily and monthly voltage baseline quality control (QC) checks were conducted throughout the course of the study. These checks allowed for the Navios settings to be standardized across different days and for different patient samples. All experiments were conducted using the EuroFlow Navios flow cytometer software (Beckman Coulter Inc. Brea, CA, USA) settings and compensation standard operating procedures (SOP) (Kalina et al, 2012).
2.2.2 EuroFlow standardised laboratory procedure

All laboratory tests for this study were conducted under the BioFlow-II laminar flow safety cabinet (Laboratory and Air Purification Systems, JHB, SA) to ensure the safety and maintenance of the sterile environment.

2.3 Detailed Instrument Set up Procedure

2.3.1 Setting up forward scatter (FS) and Side scatter (SS) parameters (Navios Acquisition software)

Fifty microliters of peripheral blood (PB) was pipetted into a 5ml FACS Tubes (BD Falcon, BD Biosciences, MA, USA). Two millilitres of freshly diluted BD FACS Lysing solution (BD Biosciences, San Jose, CA, USA) was added and the solution was mixed gently. The solution was then incubated for 10 minutes at room temperature (RT) and centrifuged for 5 minutes at 540x g using the Allegra™ X-22 centrifuge (Beckman Coulter Inc. USA). The supernatant was aspirated using a Pasteur pipette without disturbing the cell pellet, leaving approximately 50ul residual volume in the tube. The solution was mixed gently and 2ml of filtered wash buffer (See Addendum 1) was added to the tube. The cell suspension was centrifuged for 5 minutes at 540 x g. The supernatant was aspirated using a Pasteur pipette without disturbing the cell pellet and leaving approximately 50ul of the residual volume in the tube. 350ul of filtered wash buffer was added to the tube and the cells were gently resuspended. The prepared cell suspension was loaded onto the Navios flow cytometer instrument (Beckman Coulter Inc. Brea, CA, USA) for cell acquisition.

2.3.2 Gating strategy

In the Navios software (Beckman Coulter Inc. Brea, CA, USA), the Setup Mode was enabled in the Cytometer Control window. Lymphocytes were then gated in the forward scatter (FS) versus side scatter (SS) bivariate dot plot. FS and SS voltages were adjusted/fine-tuned to reach the following mean target values for gated lymphocytic population (Figure 2.2 below):

FS: 215 (range 195-235); SS: 50 (range 45-55); FS Discriminator: 90
Using the set parameters, flow cytometry data was acquired and recorded up to 10 000 events. The mean FS and SS target values for gated lymphocytes were verified and stored.

2.3.2 Setting up of PMT voltages for target fluorescence channels (Navios Acquisition software)

Mean Fluorescence Intensity (MFI) of the SPHERO Rainbow Calibration beads (Spherotech, Lake Forest, IL, USA) was used to set photomultiplier tube (PMT) voltages for each fluorescence channel. On the Navios flow cytometry software, a new protocol was created and labelled ‘8-peak Rainbow beads Target channels.’ Cytometer controls window was opened and labels were renamed according to Euroflow nomenclature (Appendix 1). In the parameters section of the software, FL3 and FL7 laser channels were disabled followed by the enabling of peak and Integral for FS and SS parameters (see Figure 2.3). Acquisition time was set to 300 seconds and maximum events was set to 10 000.
In the Analysis section of the Navios flow cytometry software, TrueView Setup was opened. In the TrueView Configuration, Trueview was enabled and TrueView Set1 was selected with axis scale changes to 10E0 -10E6.

Two dot plots FS versus SS, FITC versus PE were created alongside eight histograms, one for each fluorescence detector. In the Navios FC software, File was selected and Edit FCS Header Attributes was selected and parameter Stain names were changed to suit the EuroFlow nomenclature. The statistics showing the reference peak values x-Median (MFI) and Half Peak Coefficient of Variation (HPCV) for each fluorescence channel were displayed. The protocol was saved as “8-peak Rainbow beads Target channels”.

A fresh solution containing 1 drop of 8-peak Rainbow beads in 1ml of distilled water (dH2O) was prepared. The beads were mixed by gentle vortexing. Without recording (setup mode), the beads in solution were acquired on the Navios flow cytometer instrument at “LOW” flow rate. A region was created around the singlet beads-population P1 in the FS versus SS bivariate dot plot (Figure 2.4).
The 7th peak was gated—population 2 in the FITC versus PE bivariate dot plot after population 1 (P1) had been selected as the parent population for the dot plot (Figure 2.4). The protocol was saved for further future analyses.

![Image](https://scholar.sun.ac.za)

**Figure 2.4: Gating Singlet beads-population P1 and 7th Peak.**

The acquisition of the 8-peak Rainbow bead suspension was continued and PMT voltages were fine tuned in all fluorescence channels to reach the EuroFlow Navios target MFI values (Table 2.1).
Table 2.1: Target MFI values for the 7th peak (Trueview Settings Set 1) for the Navios flow cytometer instrument. Extracted from Kalina et al. 2015.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Fluorochrome</th>
<th>Lower MFI (-15%)</th>
<th>Target MFI for Rainbow 8-peaks</th>
<th>Upper MFI (+15%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL9</td>
<td>PacB</td>
<td>379,874</td>
<td>446,911</td>
<td>513,948</td>
</tr>
<tr>
<td>FL10</td>
<td>PacO</td>
<td>278,720</td>
<td>327,906</td>
<td>377,092</td>
</tr>
<tr>
<td>FL1</td>
<td>FITC</td>
<td>117,719</td>
<td>138,493</td>
<td>159,267</td>
</tr>
<tr>
<td>FL2</td>
<td>PE</td>
<td>101,813</td>
<td>119,780</td>
<td>137,747</td>
</tr>
<tr>
<td>FL4</td>
<td>PerCPCy5.5</td>
<td>253,075</td>
<td>297,735</td>
<td>342,395</td>
</tr>
<tr>
<td>FL5</td>
<td>PECy7</td>
<td>22,648</td>
<td>26,645</td>
<td>30,642</td>
</tr>
<tr>
<td>FL6</td>
<td>APC</td>
<td>637,655</td>
<td>750,182</td>
<td>862,709</td>
</tr>
<tr>
<td>FL8</td>
<td>APCH7</td>
<td>162,326</td>
<td>190,972</td>
<td>219,618</td>
</tr>
</tbody>
</table>

PacB: Pacific Blue, OC515: Orange Cytognos 515, alternative for FL10 was PacO, FITC: fluorescein isothiocyanate, PE: phycoerythrin, PerCPCy5.5: Peridinin-chlorophyll-proteincyanin5.5, Cy7: cyanin7, APCC750: alternative for APCH7.

A record of 2000 events was done by unclicking the setup mode and the MFI for the 7th peak of the beads was verified. PMT voltages were corrected where necessary and the recording of 2000 events redone until target MFI values were reached for all fluorescence channels. The protocol was saved. For records, the “Target MFI” and instrument settings screen was saved as .jpg picture. The PMT values and “target MFI” values were verified to be correct.

2.3.3 Fluorescence Compensation Settings

Spectral overlap in flow cytometry occurs whereby fluorescence emitted by one fluorochrome is picked up by a wrong detector. This happens due to certain fluorochromes having spectral emissions that overlap. Compensation is the mathematical process of correcting spectral overlap (also known as spill over) in multi-parametric flow cytometric data. The goal for compensation is to correct for the spill overs of all fluorescent dyes into all detectors to confirm that each parameter contains information from only a single dye.
The compensation was done after the Target MFI settings and Scatter settings (FS and SS) had been defined and established. One 5ml FACS tube was labelled as 'blank/unstained' and only 50ul of PB was pipetted into this tube. Eight more tubes were labelled according to the antibody-fluorochrome that were added per tube. These reagents were CD20-PacB, CD45-PacO, CD8-PE, CD5-PerCPCy5.5, CD8-APC, CD19-PECy7 and CD38-APCH7 (Table 2.2). To each tube, the corresponding titrated antibody and 50ul of blood were added and gently mixed. Thereafter, washing buffer was then added to reach a final volume of 100ul per tube followed by gentle mixing. The solutions were incubated protected from light at RT for 15 minutes. Two millilitres of freshly diluted 1X BD FACS lysing solution (BD Biosciences, San Jose, CA, USA) was added to the flow tube and the solution was gently mixed. The tubes were then incubated in the dark for 10 minutes before centrifugation at 540 x g. After centrifugation, the supernatant was aspirated using a Pasteur pipette leaving approximately 50ul residual volume in each tube before mixing gently. Two millilitres wash buffer was then added to each tube followed by centrifugation at 540 x g. The supernatant was aspirated using a Pasteur pipette leaving approximately 50ul residual volume in each tube filled by the addition of 350ul wash buffer to each tube before gently mixing.

Table 2.2: Antibody panel used for compensation.

<table>
<thead>
<tr>
<th>Fluorescence detectors</th>
<th>Antibody fluorochrome</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL1</td>
<td>CD8-FITC</td>
<td>Cytognos</td>
</tr>
<tr>
<td>FL2</td>
<td>CD8-PE</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td>FL4</td>
<td>CD19-PECy7</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td>FL5</td>
<td>CD5-PerCPCy5.5</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>FL6</td>
<td>CD8-APC</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td>FL8</td>
<td>CD38-APCH7</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>FL9</td>
<td>CD20-PacB</td>
<td>Biolegend</td>
</tr>
<tr>
<td>FL10</td>
<td>CD45-PacO</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>
2.3.4 Flow cytometry Immunophenotyping

The EuroFlow standardised 8 colour flow cytometry panel for B-cell chronic lymphoproliferative disorders (B-CLPD) was used for the identification of CLL. EuroFlow lymphoid screening tube (LST) immunophenotyping panel (Table 2.3) with antibody markers CD20, CD4, CD45, CD8, smlgλ, CD56, smlgκ, CD5, CD19, TCRγδ, smCD3 and CD38. Tube 2 of EuroFlow B-CLPD panel, combined with LST, can identify CLL from other B-CLPD and included CD20, CD45, CD23, CD10, CD79b, CD19, CD200 and CD43. Flow cytometry data from Navios was further analysed using Infinicyt™ flow cytometry software. Immunophenotypic profiles from CLL positive patients will then be stored in a database using the compass tool of the Infinicyt™ FC software.

Table 2.3. Euroflow limited B-CLPD panel including LST panel. Adapted from van Dongen et al. 2012.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Pacific blue</th>
<th>Pacific orange</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP-Cy5.5</th>
<th>PE-Cy7</th>
<th>APC</th>
<th>APC-H7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CD 20 and CD4</td>
<td>CD45 and CD8 and smlgλ</td>
<td>CD45 and CD56 and smlgκ</td>
<td>CD5</td>
<td>CD19 and TCRγδ</td>
<td>smCD3</td>
<td>CD19</td>
<td>CD200</td>
</tr>
<tr>
<td>2</td>
<td>CD20</td>
<td>CD45</td>
<td>CD23</td>
<td>CD10</td>
<td>CD79b</td>
<td>CD19</td>
<td>CD200</td>
<td>CD43</td>
</tr>
</tbody>
</table>

FITC: Fluorescein isothiocyanate; PE: Phycoerythrin; Cy5: cyanin5; Cy7: Cyanin7; APC: Allophycocyanin; PerCP: Peridinin-chlorophyll-protein; H7: Hilite; Backbone are highlighted in yellow

2.4 Sample preparation and staining for flow cytometric data acquisition

Euroflow LST and B-CLPD (Table 2.4) fluorochrome combinations were used for flow cytometric analysis. Euroflow 8-colour antibody panels were prepared for flow cytometry analysis using the detailed Euroflow Standard Operating Procedures (SOPs) for sample preparation and staining (www.euroflow.org) (van Dongen et al. 2012)
Table 2.4: List of antibody volume per test, manufacturer, and clone.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Volume (ul)</th>
<th>Fluorochrome</th>
<th>Clone</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>smCD3</td>
<td>2.5</td>
<td>APC</td>
<td>SK7</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>CD38</td>
<td>3</td>
<td>APCH7</td>
<td>HB7</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>CD43</td>
<td>2.5</td>
<td>APCH7</td>
<td>IG10</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>CD79b</td>
<td>10</td>
<td>PerCPCy5.5</td>
<td>SN8</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>CD19</td>
<td>5</td>
<td>PC7</td>
<td>J3-119</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td>CD10</td>
<td>20</td>
<td>PE</td>
<td>ALB1</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td>TCRyδ</td>
<td>3</td>
<td>PC7</td>
<td>11F2</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>CD 4</td>
<td>0.5</td>
<td>PacB</td>
<td>RPA-T4</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD8 and smlgλ</td>
<td>20 (part of mix)</td>
<td>FITC</td>
<td>UCH-T4</td>
<td>Cytognos</td>
</tr>
<tr>
<td>CD56 and smlgk</td>
<td>20 (part of mix)</td>
<td>PE</td>
<td>C5.9</td>
<td>Cytognos</td>
</tr>
<tr>
<td>CD200</td>
<td>1.25</td>
<td>APC</td>
<td>OX104</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD23</td>
<td>2.5</td>
<td>FITC</td>
<td>MHM6</td>
<td>Dako</td>
</tr>
<tr>
<td>CD20</td>
<td>1</td>
<td>PacB</td>
<td>2H7</td>
<td>Biologend</td>
</tr>
<tr>
<td>CD45</td>
<td>5</td>
<td>PacO</td>
<td>HI30</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>CD5</td>
<td>15</td>
<td>PerCPCy5.5</td>
<td>L17F12</td>
<td>BD Bioscience</td>
</tr>
</tbody>
</table>

Three hundred microliters of peripheral blood from EDTA tube were aliquoted into a 10ml Falcon tube. Six millilitres of washing buffer were added to the tube and mixed well. Four millilitres of washing buffer were added to filled up the tube to 10ml and the solution was mixed well. The tube was centrifuged for 5 minutes at 540 x g and the supernatant was removed and discarded using a Pasteur pipette without disturbing the cell pellet. Following gentle mixing, another 6ml washing buffer was added again and the centrifugation and supernatant removal was repeated two more times. The solution was than centrifuged at 540 x g for 5 minutes and the supernatant was discarded using a Pasteur pipette without disturbing the cell pellet before mixing well. Thereafter, the cell pellet was suspended in 300ul of washing buffer before continuing to the next phase of the EuroFlow SOP (Kalina et al. 2012).
2.4.1 Staining of back bone markers – Tube 2 only

The stipulated volumes of cell surface membrane backbone markers CD19, CD20 and CD45 (Table 2.4) were multiplied by 2 and the result volumes of each antibody marker were pipetted into one 5ml FACS tube (backbone tube). Thereafter, the total volume of sample to be stained was calculated based on the number of tubes in the immunophenotype panel a volume of 50ul PB per tube. We utilised two tube panel therefore we multiplied the 50ul PB volume by 2 resulting in a total volume of 100ul PB pipetted into the backbone tube. The mixture was mixed well using a pipette. The final backbone mix was then split into 2 equal aliquots which were subsequently pipetted into two separate 5ml FACS tubes marked Tube 1 and Tube 2 respectively.

2.4.2 Staining of surface markers

An appropriate number of antibodies (Table 2.1) directed against cell surface markers were added to a tube. The backbone markers were excluded at this point. Washing buffer was added to reach a final volume of 100ul per in both tube 1 and tube 2, mixing the contents in each tube well using a pipette. The two tubes were then incubated in the dark for 30 minutes at RT. Thereafter, 2ml of 1X FACS lysing solution (BD Biosciences, MA, USA) was added to tube 1 and tube 2 before mixing well. The two tubes were further incubated in the dark for 10 minutes at RT followed by centrifugation for 5 minutes at 540 x g. After centrifugation, the supernatant was discarded using a Pasteur pipette. Care was taken not to disturb the cell pellet and approximately 50ul residual volume was left in each tube. The 50ul residual volume and pellet were and re-suspended in 200ul of PBS (Sigma Aldrich, MO, USA). The cells were preferably immediately acquired after staining or stored at 4°C maximally for 1 hour until measurement on the flow cytometer.
2.5 Gating Strategy

A total of 50 000 cells were acquired for both tube 1 and tube 2 in the EuroFlow limited BCLPD panel for CLL. The cells from the two tubes were combined for analysis by the Infinicyt flow cytometry software (Cytognos, Salamanca, ESP) to give a total of 100 000 cells in a single analysis. The analysis strategies for the two methods were generally similar (Figures 3.1 A and B) except for their unique markers which are recommended by expert consensus but are not critical to the diagnosis of CLL.
Figure 2.5: EuroFlow and NHLS gating strategies, including used antibody markers. (A) EuroFlow CLL panel gating strategy (B) NHLS CLL panel gating strategy. For both low SS, FS and CD45 are used to gate on the lymphocytes, follow by B-cells isolation via low SS, CD45 and CD19. Clonality are identified using kappa and/or lambda. The CLL identifying markers are similar except for CD79b and CD43 in the EuroFlow panel and FMC7 and CD22 in the NHLS panel (Those highlighted in yellow). Furthermore, CD38 are used in both panels as a prognostic marker. The unique markers in each of the flow cytometry panels are highlighted in yellow.

The cells were initially gated on the SS versus FS area to discriminate between the lymphocytes and the rest of the cells (Figure 2.6A). Thereafter, CD45 positive cells were gated against low SS to identify the lymphocyte population. The cells were also gated on the FS area versus FS height to discriminate between the single cell populations and the doublet populations (Figure 2.6B).
Figure 2.6: Representative illustration of lymphocyte gating. (A and C) show gating of all leukocytes on the SS and FS and CD45 dot plot diagrams. (B) doublet discrimination. (D and E) show gating for lymphocytes on the SS and FS and CD45 low SS cells.
The identified CD45 positive lymphocytes were further gated using low SS versus CD19+ and CD20+ to identify the B-cells (Fig 2.5 and 2.7).

**Figure 2.7: Representative illustration of B-cells gating from the lymphocytes.** (A) B-cells were gated as low SS and CD19+. (B) Shows clonal kappa cells and restriction of lambda cells, typical phenotype in CLL malignancy.

The B-cells were further interrogated using Tube 2 markers to determine the CLL immunophenotype (Table 1.1, 2.3 and 2.4). Expression of Kappa and Lambda markers by B-cells was noted and restricted expression of either of the two markers is a phenotype in keeping with the diagnosis of CLL (Figure 2.7).

The Co-expression of CD5, CD19 and CD23 coupled with the positive expression of CD200 and CD43 signified the presence of CLL. Furthermore, CD10 was expected to be negative and low or no expression CD79b to identify CLL. CD38 was a prognostic marker.
2.5.1 Infinicyt™ software

The Infinicyt version 1.8 flow cytometry analysing software (Cytognos, Salamanca, ESP) was used to analyse the EuroFlow data. The Compass tool Infinicyt software was used to create a database of CLL positive cohorts at TAH. The compass tool allows for the automatic separation of different disease entities based on their immunophenotypic profiles. This separation is facilitated by the Automatic Population separator tool (APS).
2.3.7 Cost Analysis

A cost analysis of the reagents used was drawn up for the immunophenotype panels of the EuroFlow and NHLS method.

2.6 Incidence of chronic lymphocytic leukaemia at Tygerberg Academic Hospital over a 6-year period, 2011 to 2016

To describe trends in CLL at Tygerberg Hospital, records of all patients who were diagnosed with CLL at Tygerberg hospital from the year 2011 to 2016 were analysed (Figure 2.8). This was done via the patient record keeping software DISALAB (v04.16.04.814, Laboratory System Technologies, Johannesburg, SA) for the years 2011-2015 and TrakCare (L6.10, InterSystems Corporation, Cambridge, MA, USA) for the years 2015 and 2016.

Figure 2.8: Workflow for data collection, CLL diagnosis confirmation and grouping of data according to gender, age, HIV, and cytogenetic properties.
Each patient’s file was investigated to verify the diagnosis of CLL. The CLL diagnosis was verified by a haematopathologist from the Tygerberg Hospital NHLS haematology division. Thereafter, their gender of was noted and recorded. The same procedure also was done for age, HIV status and cytogenetics for every patient.

2.6.1 Statistical analysis

All statistical analyses were performed in consultation with a statistician. Statistical analyses were done using GraphPad Prism software (Version 5, GraphPad Software, San Diego, California, USA). The following details were captured and analysed using descriptive statistics; Age, gender, HIV status and cytogenetic characteristics. The age and gender data was tested for normality using the Kolmogorov-Smirnov test. The two-tailed independent t test with 95% confidence interval was used to describe the trends in CLL at Tygerberg Hospital.
CHAPTER 3: RESULTS

This study was divided into two parts. The first part was the assessment of the EuroFlow standardised multicolour flow cytometry panel and protocols for immunophenotyping and subsequently the construction of an immunophenotyping database at TAH consisting of locally generated CLL flow cytometry profiles. The second part of the study was describing CLL incidence trends at TAH based on number of cases, age, gender, HIV, and molecular (cytogenetics) as there is a lack of and/or a paucity of CLL data within our setting.

3.1 Standardised multicolour flow cytometry study

3.1.1 Population description of CLL participants

Twenty voluntary CLL patients participated in the flow cytometry based sub-study. Table 3.1 in chapter 3 summarised the CLL patient’s demographics. Of the 20 patients, 35 % (7) were female compared to the 65% (13) male proportion. The ages of the participating patients ranged from 47 to 84 years of age. The total mean age was 65 years, with a 71 year mean for the females and 62 years mean for the male. Table 3.1 below summarises all the demographics of the voluntary participating CLL patients taking part of the flow cytometry based sub-study.
### Table 3.1: Demographic data of participating CLL patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total participants</td>
<td>20</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>7(35)</td>
</tr>
<tr>
<td>Male</td>
<td>13(65)</td>
</tr>
<tr>
<td><strong>Age</strong> Years</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>47-84</td>
</tr>
<tr>
<td>Total mean</td>
<td>65</td>
</tr>
<tr>
<td>Mean female</td>
<td>71</td>
</tr>
<tr>
<td>Mean male</td>
<td>62</td>
</tr>
</tbody>
</table>

### 3.1.2 Flow cytometry analysis

This study assessed the use of EuroFlow standardised flow cytometry protocols and immunophenotype panels in the TAH catchment area and used CLL as a proof of principle concept. The goal was to validate and ascertain whether the EuroFlow multicolour panels are suitable for the diagnosis of haematological malignancies in the South African population. In addition, the study aimed to construct a local immunophenotype database containing flow cytometry profiles of CLL cases at TAH.

The standardised and validated EuroFlow protocols and panels (Table 3.2) were evaluated and compared to the current NHLS flow cytometry setup and panels for CLL. Comparisons were made in terms of the instrumentation, panel markers, gating strategy, turnaround time (TAT), analysis and cost. The results were also obtained via multicolour flow cytometry (8 colour) using the Beckman Coulter Navios while routine flow cytometry diagnosis of CLL at TAH is performed on the BD FACS Caliber 4 colour flow cytometer (BD Biosciences, SG). Results from the EuroFlow standardised 8 colour flow cytometry method correlated well with the 4 colour NHLS method in 20/20 (100%) patients.
Table 3.2: EuroFlow limited B-CLPD (A) and NHLS flow cytometry panels (B) for CLL. Markers unique to each panel are highlighted in bold. Adapted from van Dongen et al. 2012.

(A) EuroFlow Tube

<table>
<thead>
<tr>
<th>Tube</th>
<th>PacB</th>
<th>PacO</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP-Cy5.5</th>
<th>PE-Cy7</th>
<th>APC</th>
<th>APC-H7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (LST)</td>
<td>CD 20*</td>
<td>CD45*</td>
<td>CD8</td>
<td>CD5</td>
<td>CD56</td>
<td>CD5</td>
<td>CD19*</td>
<td>smlCD3</td>
</tr>
<tr>
<td></td>
<td>CD4</td>
<td></td>
<td>smlgλ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>CD20*</td>
<td>CD45*</td>
<td>CD23</td>
<td>CD10</td>
<td>CD79b</td>
<td>CD19*</td>
<td>CD200</td>
<td>CD43</td>
</tr>
</tbody>
</table>

(B) NHLS Tube

<table>
<thead>
<tr>
<th>Tube</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP</th>
<th>APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CD8</td>
<td>CD4</td>
<td>CD45</td>
<td>CD3</td>
</tr>
<tr>
<td>2</td>
<td>CD5</td>
<td>CD23</td>
<td>CD45</td>
<td>CD19</td>
</tr>
<tr>
<td>3</td>
<td>CD20</td>
<td>CD10</td>
<td>CD45</td>
<td>CD38</td>
</tr>
<tr>
<td>4</td>
<td>FMC-7</td>
<td>CD22</td>
<td>CD45</td>
<td>CD200</td>
</tr>
<tr>
<td>5</td>
<td>L</td>
<td>K</td>
<td>CD45</td>
<td>CD19</td>
</tr>
</tbody>
</table>

sm=surface membrane, λ = lambda, k=kappa, y=gamma, δ=delta, LST = lymphoid screening tube, markers highlighted in yellow are unique to each flow cytometry panel, respectively. FITC: Fluorescein isothiocyanate; PE: Phycoerythrin; Cy5: cyanin5; Cy7: Cyanin7; APC: Allophycocyanin; PerCP: Peridinin-chlorophyll-protein; H7: Hilit;

*=EuroFlow B-CLPD panel backbone markers

3.1.3 Antibody panels and analysis gating strategies

The EuroFlow and NHLS flow cytometry panels (Table 3.2) had mostly similar antibody markers except for 6 markers. The EuroFlow panel had antibody markers CD79b, CD43, CD56 and TCRγδ which were not included in the NHLS panel. However, out of the four, only CD43 and CD79b were directly functional in CLL diagnosis (McCarron et al. 2000; Falay et al. 2017). CD56 and TCRγδ are respectively NK cell and T cell lineage identifiers (van der Velden et al. 2017). On the other hand, the NHLS included CD22 and FMC7 markers which were unique to their panel.
Both the EuroFlow and NHLS markers (Table 3.2) could be reduced to a few tubes to specifically identify cell lineages. Tube 1 of the EuroFlow panel, namely the LST tubes can identify T, B and NK cells. Two of the 5 tubes within the NHLS panel can directly identify between B-cells and T cells. NK cells are only identified by exclusion due to the absence of CD56 marker within the NHLS panel. In addition, for the NHLS panel, CD8 positive cells that are CD3 negative can be identified as NK cells. Unlike EuroFlow, NHLS lineage tubes could not identify the gamma delta T cells because of the absence of the TCRyδ marker in the panel.

In both EuroFlow and NHLS, CLL diagnoses were made when markers identified as CD5+, CD23dim/+, CD20dim/+, CD19+, CD45+, CD200+ and CD10- with either kappa or lambda clonality based on their expression levels (Figure 3.1 and Figure 3.2). In addition, the EuroFlow panel required CD43+ and CD79b dim/- whilst the NHLS panel required FMC7dim/- and CD22dim/+.
Figure 3.1: Representative diagram from one of the analysed samples illustrating the typical CLL immunophenotype profile using the EuroFlow flow cytometry panels. (A1) Lymphocytes gated on low side scatter (SS) and positive CD45. (B1) B-cells are then gated on low SS and CD19 population of lymphocytes. The clonal B-cells were assessed further (C1) Clonal assessment of B-cells using Ig lambda and Ig kappa; dot plot shows the B-cells are clonal for kappa, (A2) CD23+, (B2) CD19+CD5+, (C2) CD5+CD20dim (A3) CD19+CD10+(B3) CD19+CD79b dim/- B-cells. (C3) CD19+CD43+.
Figure 3.2: Representative diagrams from one of the analysed sample illustrating the typical CLL immunophenotype using the NHLS flow cytometry panels. Cells of interest are highlighted blue; (A1) Lymphocytes show low FS and low SS, (B1) Lymphocytes show low FS and CD45+, (C1) Low SS and CD19+ B-cells gated from lymphocytes. The B-cells were assessed further; (A2) Clonal assessment of B-cells using Ig lambda and Ig kappa; dot plot shows the B-cells are clonal for kappa, (B2) CD20+CD10- (C2) CD5+CD23+ (A3) CD22+CD200+. 
HIV and CLL co-infection: Interestingly, one patient had concurrent CLL and HIV infection. This patient had a typical CLL immunophenotype that was confirmed by both the NHLS and EuroFlow panels. Previous studies have reported that this is a rare occurrence and HIV course might be unrelated to CLL disease (Shimada et al. 2015).

3.1.4 Establishment of CLL immunophenotype database

Following confirmation of the CLL using the EuroFlow panels, the 20 CLL cases were grouped into an immunophenotype database (Figure 3.3) using another feature of the Infinicyt flow cytometry analysis software. The database produced an immunophenotype map for the 20 CLL cases (Figure 3.4 A and B) showing the immunophenotype area where CLL cases at TAH fall within. To our knowledge, the database is the first of its kind representing standardised CLL disease immunophenotype at TAH.
Figure 3.3: Representation of population parameters for patients added to the Tygerberg Academic Hospital CLL database. The diagram shows where the markers for all the CLL patients are concentrated in the TAH database. The overall immunophenotype clearly represents that of typical CLL.
Figure 3.4: Diagrams represent CLL immunophenotype database at Tygerberg Academic Hospital. (A) Immunophenotype map of CLL cases at TAH. Black area shows new cases being added to the database. The red area represents grouped database cases. Some CLL cases were clonal for lambda while some were clonal for kappa. (B) Diagram represents an immunophenotype map resulting from the grouped CLL cases in the database. The black area shows the new CLL case that has been added to the database. The red dots represent grouped database cases. The arrow shows an isolated CLL case which may represent atypical CLL in the database.
Cost of analysis of marker panels

Cost analysis of the two flow cytometry methods was also performed and showed that the EuroFlow standardised multicolour panel was cheaper than the NHLS panel by ZAR 0.06 (Table 3.3).

Table 3.3: Cost analysis of NHLS and EuroFlow flow cytometry panels for the diagnosis of CLL

<table>
<thead>
<tr>
<th>Marker</th>
<th>Cost per antibody</th>
<th>NHLS panel</th>
<th>Marker</th>
<th>Cost per antibody</th>
<th>EuroFlow panel</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8 FITC</td>
<td>R</td>
<td>55.60</td>
<td>C20 PacB</td>
<td>R</td>
<td>51.88</td>
</tr>
<tr>
<td>CD4 PE</td>
<td>R</td>
<td>80.11</td>
<td>CD4 PacB</td>
<td>R</td>
<td>11.38</td>
</tr>
<tr>
<td>CD45PerCP</td>
<td>R</td>
<td>230.26</td>
<td>CD45 PacO</td>
<td>R</td>
<td>88.54</td>
</tr>
<tr>
<td>CD3APC</td>
<td>R</td>
<td>39.33</td>
<td>CD8 and smlgλ FITC/CD56 and smlgk PE</td>
<td>R</td>
<td>310.85</td>
</tr>
<tr>
<td>CD5 FITC</td>
<td>R</td>
<td>55.60</td>
<td>CD23 FITC</td>
<td>R</td>
<td>10.39</td>
</tr>
<tr>
<td>CD19 APC</td>
<td>R</td>
<td>126.50</td>
<td>CD10 PE</td>
<td>R</td>
<td>10.08</td>
</tr>
<tr>
<td>CD20 FITC</td>
<td>R</td>
<td>49.29</td>
<td>CD5 PerCP-Cy5.5</td>
<td>R</td>
<td>68.56</td>
</tr>
<tr>
<td>CD10 PE</td>
<td>R</td>
<td>103.28</td>
<td>C79b PerCP-Cy5.5</td>
<td>R</td>
<td>64.81</td>
</tr>
<tr>
<td>CD38 APC</td>
<td>R</td>
<td>89.09</td>
<td>CD19 PECy7</td>
<td>R</td>
<td>15.21</td>
</tr>
<tr>
<td>FMC7 FITC</td>
<td>R</td>
<td>113.60</td>
<td>TCRγδ PECy7</td>
<td>R</td>
<td>168.52</td>
</tr>
<tr>
<td>CD22 PE</td>
<td>R</td>
<td>53.53</td>
<td>smCD3 APC</td>
<td>R</td>
<td>25.28</td>
</tr>
<tr>
<td>CD200 APC</td>
<td>R</td>
<td>76.88</td>
<td>CD200 APC</td>
<td>R</td>
<td>133.78</td>
</tr>
<tr>
<td>Kappa</td>
<td>R</td>
<td>35.25</td>
<td>CD38 APCH7</td>
<td>R</td>
<td>101.52</td>
</tr>
<tr>
<td>lambda</td>
<td>R</td>
<td>42.77</td>
<td>CD43 APCH7</td>
<td>R</td>
<td>84.26</td>
</tr>
<tr>
<td>Total cost per test</td>
<td>R</td>
<td>1 151.10</td>
<td>Total cost per test</td>
<td>R</td>
<td>1 145.04</td>
</tr>
</tbody>
</table>
3.2 Incidence of CLL within the Tygerberg Academic Hospital Service Area

These results represent the second part of our study which describes CLL trends in TAH over a 6-year period from the year 2011 to 2016. To our knowledge this is the first study of its kind at TAH.

All patients in this study are confirmed CLL cases. Diagnosis was confirmed by clinical Haematology and NHLS pathologists following confirmation via laboratory testing. As the hospital has an internal registry system for all cases of cancer, one could collate required data for the CLL cases since 2011-2016. Characteristics of all CLL cases captured over the 6-year period are summarised within Table 3.4.

Table 3.4: Characteristics of TAH patients with newly diagnosed CLL from 2011 to 2016

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total patients</td>
<td>80</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>39 (48.8)</td>
</tr>
<tr>
<td>Women</td>
<td>41 (51.2)</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>67</td>
</tr>
<tr>
<td>Interquartile range</td>
<td>60 - 74</td>
</tr>
<tr>
<td>Range</td>
<td>37 - 95</td>
</tr>
<tr>
<td>&lt;40</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>41-49</td>
<td>6 (7.5)</td>
</tr>
<tr>
<td>50-59</td>
<td>13 (16.3)</td>
</tr>
<tr>
<td>60-69</td>
<td>29 (36.3)</td>
</tr>
<tr>
<td>70-79</td>
<td>17 (21.3)</td>
</tr>
<tr>
<td>80-89</td>
<td>13 (16.3)</td>
</tr>
<tr>
<td>90&gt;</td>
<td>1 (1.3)</td>
</tr>
</tbody>
</table>

The incidence of CLL at TAH appears to follow an erratic pattern (Figure 3.5). The incidence is generally similar between men and women (Table 3.4) which deviates from what has been reported elsewhere in the world where CLL incidence is slightly higher in males than females (Surveillance Research Program - NCI 2014).
Figure 3.5: CLL incidence distribution was erratic, changing yearly with no defined pattern. Red line illustrates the erratic incidence pattern.

However, the trend of new CLL diagnoses at TAH appeared to be slightly higher in women than in men although there was no significant difference between the two groups (p value = 0.93).

The median age at diagnosis at TAH is 67 years (Table 3.7). This is in agreement with reports from the Western countries including the United States of America and England (Parikh et al. 2013). The modal age range for diagnosis at TAH was 60 to 74 years (Figure 3.6 A and B).
Figure 3.6: CLL distribution by age at Tygerberg Academic Hospital. (A) Age range of CLL patients at diagnosis and (B) Box and whisker plot showing the modal age range at diagnosis between 60 and 74 years at Tygerberg Academic Hospital.
Yearly comparison of age at diagnosis between men and women did not appear to be influenced by gender (Figure 3.7). However, an overall comparison of age at diagnosis between men and women showed that at TAH, men are generally diagnosed with the disease at an earlier age than women (Figure 3.8). There was a significant difference between the overall age of diagnosis between men and women ($p=0.023$) at TAH from the year 2011 to 2016.

![Yearly distribution of incidence by gender](image-url)

Figure 3.7: Yearly average age of men and women at diagnosis of CLL at Tygerberg Academic Hospital.
Few cases (3/80) of concurrent CLL and HIV infection were observed (Figure 3.9). The CLL immunophenotype of these patients was that of typical CLL, the same as those who did not have HIV. Generally, patients over the age of 65 were not tested for HIV infection. In total, 3 (6%) patients tested positive for HIV infection out of the 48 patients that were tested. However, it is important to note that it is not known whether they had HIV infection prior to the diagnosis of CLL or not.

The 3 patients with both HIV and CLL appeared to have the same disease course as patients with CLL that were negative for HIV. They had typical immunophenotypes for CLL disease suggesting that HIV does not change the typical immunophenotype profiles that are expected in these patients. In addition, the cytogenetic characteristics followed no particular trend as they showed the same cytogenetic abnormalities as the HIV negative patients with CLL. Our results agree with previous literature that reported that the co-occurrence of CLL and HIV is rare (Shimada et al., 2015).
Figure 3.9: Representation of distribution of CLL by HIV infection at Tygerberg Academic Hospital.
3.2.1 Cytogenetic profiling

In the TAH, cytogenetic assessments (Table 3.5), were mainly done using the FISH technique however, conventional karyotyping was used for a few patients (6/80).

Table 3.5: Prognostic cytogenetics profile of patients diagnosed with CLL at TAH from 2011 to 2016

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. (%)</th>
<th>No. Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Del13q14</td>
<td>4 (22)</td>
<td>18</td>
</tr>
<tr>
<td>Del13q34</td>
<td>all negative</td>
<td>7</td>
</tr>
<tr>
<td>Trisomy 12</td>
<td>7 (33)</td>
<td>21</td>
</tr>
<tr>
<td>Del17p13</td>
<td>all negative</td>
<td>20</td>
</tr>
<tr>
<td>Del11q22</td>
<td>5 (24)</td>
<td>21</td>
</tr>
<tr>
<td>t(11;14)</td>
<td>all negative</td>
<td>8</td>
</tr>
<tr>
<td>t(8;14)</td>
<td>all negative</td>
<td>1</td>
</tr>
<tr>
<td>t(14;18)</td>
<td>all negative</td>
<td>4</td>
</tr>
</tbody>
</table>

From 2011 to 2016, most of the patients at TAH were not assessed for cytogenetic abnormalities. Of the cohort that was assessed, selected cytogenetic tests were administered (Table 3.5 and Figure 3.10). In total, 37% (30/80) patients were assessed for cytogenetic abnormalities and of those, different tests were performed.

The presence of the deletion 13q14 was assessed in a total of 18 patients of which only 22% (4/18) were found to be carrying the defect. Deletion 13q34 was tested in 7 patients and all of them had negative results.

Trisomy 12 was assessed in a total of 21 patients and 33% (7/21) of the patients were reported positive for the abnormality. Equally, 21 patients were also assessed for deletion 11q22 abnormality and 24% (5/21) carried the chromosomal abnormality. Of the 21, 2 patients, a female aged 50 years and a male aged 57 years were observed to have been carrying both the trisomy 12 and the 11q22 abnormalities.
Deletion 17p13 was assessed in a total of 20 patients. All the 20 patients fell under the same group of individuals who had been assessed for deletion 11q22. None of the 20 patients tested positive for the deletion 17p13 abnormality. The two abnormalities appeared to be mutually exclusive for the population at TAH.

Combined, translocation t(11;14), t(4;18) and t(14;18) were assessed in 13 patients. None of the CLL patients diagnosed at TAH between 2011 and 2016 had the translocation abnormalities (Table 3.5).
CHAPTER 4: DISCUSSION

This thesis focussed on introducing multicolour flow cytometry in the diagnosis of haematological malignancies at TAH, SA. It also explored the idea of standardisation of immunophenotyping of haematological malignancies at TAH using EuroFlow, an independent scientific consortium’s findings. EuroFlow was established in Europe with the aim of innovating and standardising flow cytometric immunophenotyping to improve diagnostic patient care. This consortium also created a data base of haematological malignancies in order to increase the sensitivity and specificity of immunophenotyping. Creating a data base of disease cases resulted in shorter turnaround times during diagnosis and require less staff for analysis. Furthermore, it assisted pathologists in diagnosing difficult cases as comparability between various institutions could now be performed as the same instruments and standard operating procedures (SOP) were implemented at all the institutions that belonged to the consortium. In addition, inter-laboratory research with larger numbers of patients studied was now possible. Various studies have identified important variables in flow cytometry sample handling, instrument setup, data analysis and reagents (McCoy & Overton 1994; Maecker et al. 2012), which further emphasizes the rationale behind this study. However, as we are in a resource poor setting we firstly had to understand if EuroFlow panels were suitable within our patients’ population and secondly, we had to compare costing and prove the cost effectiveness of introducing EuroFlow for the diagnosis of CLL at our institution.

Thus, the present investigation evaluated and assessed the EuroFlow standardised SOPs and panels for immunophenotyping CLL within our population setting via multicolour flow cytometry. Likewise, immunophenotyping database containing local CLL flow cytometry profiles at TAH were also established in order to reduce the turnaround times of future CLL flow based diagnoses. In addition, due to lack of and/or paucity of data related to CLL or specific types of leukaemias in the Southern African regions we further investigated CLL incidence trends at TAH over a 6-year period as well as the age, gender, HIV status of the patients. We also documented where applicable the type of molecular investigations that were performed.
4.1 Multicolour flow cytometry

4.1.1 Protocol, marker panel and gating strategies comparisons between EuroFlow and NHLS flow cytometry CLL based assays

The two immunophenotyping panels EuroFlow and NHLS (See Chapter 3, Table 3.1, for further details) have various important differences. The NHLS uses a 4-colour flow cytometry panel that consists of 5 tubes with a total of 16 antibody markers whereas the EuroFlow standardised multicolour flow cytometry panel uses an 8 colour, 2 tube panel with 17 antibody markers. These differences already highlight that the numerous tubes used by the NHLS panel laborious, which would most likely lead to an increase in turnaround time (TAT). Furthermore, this method would be more prone to costly human errors with downstream consequences. Some of these errors that we have experienced at the NHLS, TAH, include for instance inadvertently forgetting to add antibody markers in certain tubes, which have resulted in repeating those particular markers, which in turn would affect TAT and subsequent delays in releasing diagnostic results.

4.1.1.1 Antibody marker Panels

In addition, both the EuroFlow and NHLS panels had markers that were unique to each of them. In contrast to the NHLS panel, the EuroFlow panel had CD56 (NK cell lineage identifier), TCRyδ (T Cell lineage identifier), CD43 (activated B-cells marker) and CD79b (B-cells marker) while the NHLS had CD22 (pan B-cell marker) and FMC7 (late B-cell differentiation marker) which were interestingly absent in the EuroFlow panel. However, it is important to note that the full BCLPD panel for the EuroFlow includes these markers. The NHLS at TAH uses the diagnostic algorithm called the Matutes scoring system for CLL (Matutes et al. 1994; Moreau et al. 1997), however substituting CD22 for CD79a as they are both B-cells markers and instead of using sIg, kappa or lambda are used to identify light chain restriction.

Tube one of the EuroFlow panel, named the LST tube, had a combination of screening antibodies that identify lineages of the B-cells, T cells and NK cells (Table 4.1) cell populations. The NHLS panel could be reduced to two tubes capable of screening for
cell population lineages. In both the EuroFlow and NHLS panels, the B-cells were identified using the antibody markers CD45 (leukocyte), CD19 (pan B-cell) and CD20 (pan B-cell) (Figure 3.1 A and B and Figure 3.3 and 3.4). In both panels, CD45 is a marker of mature lymphocytes. CD3 was also used to identify B-cells by exclusion. To identify subpopulations of T cells, both panels employed CD45, CD3 (T-cell identifier), CD8 (cytotoxic T-cell) and CD4 (helper T-cell). In addition, the EuroFlow panel had a unique marker TCRγδ for gamma delta for confirmation of T cell lineage.

As mentioned earlier, the EuroFlow panel identified NK cells using the markers CD56 (Table 3.1 A and B) while the NHLS panel lacks the CD56 marker to directly identify the NK cells. However, the NHLS panel included CD3 which could be used to identify NK cells by exclusion in CD8 expressing cells. Likewise, both CD20 and CD19 could also be used to identify NK cells by exclusion. In the NHLS panel, further assessment using an extended immunophenotype panel was required to determine the NK cell presence. However, this study was concerned with B-cells and therefore both the NHLS and EuroFlow were effective for the purpose of CLL identification.
Table 4.1: Positive and negative reference lymphocyte populations used for the various markers and their gating strategy for the EuroFlow LST. Adapted from van der Velden et al. 2017.

<table>
<thead>
<tr>
<th>Target marker</th>
<th>Positive reference population</th>
<th>Gating strategy</th>
<th>Negative reference population</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8</td>
<td>CD8+ T-cells</td>
<td>CD8^{hi}/CD45^{hi}/SS^{lo}</td>
<td>CD4^{+} T-cells</td>
</tr>
<tr>
<td>IgLambda</td>
<td>Igλ+ B-cells</td>
<td>CD20^{+}/CD19^{+}/Igκ^{-}/CD3^{-}</td>
<td>CD4^{+} T-cells</td>
</tr>
<tr>
<td>IgKappa</td>
<td>Igκ+ B-cells</td>
<td>CD20^{+}/CD19^{+}/Igλ^{-}/CD3^{-}</td>
<td>CD4^{+} T-cells</td>
</tr>
<tr>
<td>CD56</td>
<td>NK-cells</td>
<td>CD3^{-}/CD19^{-}/CD20^{-}/CD45^{hi}/SS^{lo}/CD56^{+}</td>
<td>CD4^{+}/CD56^{-} T-cells</td>
</tr>
<tr>
<td>CD5</td>
<td>CD4^{+} T-cells</td>
<td>CD4^{+}/CD45^{hi}/SS^{lo}</td>
<td>B-cells</td>
</tr>
<tr>
<td>CD19</td>
<td>B-cells</td>
<td>CD20^{hi}/CD45^{hi}/SS^{lo}/CD3^{-}</td>
<td>NK-cells</td>
</tr>
<tr>
<td>TCRγδ</td>
<td>TCRγδ^{+} T-cells</td>
<td>TCRγδ^{+}/CD45^{hi}/SS^{lo}/CD3^{very high}</td>
<td>NK-cells</td>
</tr>
<tr>
<td>CD3</td>
<td>CD4^{+} T-cells</td>
<td>CD4^{+}/CD45^{hi}/SS^{lo}</td>
<td>B-cells</td>
</tr>
<tr>
<td>CD20</td>
<td>B-cells</td>
<td>CD19^{+}/CD45^{hi}/SS^{lo}/Igκ^{+} or Igλ^{+}/CD3^{-}</td>
<td>NK-cells</td>
</tr>
<tr>
<td>CD4</td>
<td>CD4^{+} T-cells</td>
<td>CD4^{+}/CD45^{hi}/SS^{lo}</td>
<td>NK-cells</td>
</tr>
<tr>
<td>CD38</td>
<td>NK-cells</td>
<td>CD3^{-}/CD19^{-}/CD20^{-}/CD45^{hi}/SS^{lo}/CD56^{+}</td>
<td>NA</td>
</tr>
</tbody>
</table>

When comparing the two SOPs, the EuroFlow LST panel was the more cost effective of the two for lineage identification as it only uses one tube in contrast to the NHLS panel. The discrimination between the 3 lymphoid lineages was possible by using the LST tube. The advantage of using this tube is the ability to distinguish reactive causes of disease from clonal disorders. This therefore assists in saving time and costs. In SA, this tube would be very useful because more patients have HIV and Tuberculosis as a
cause for lymphadenopathy and would easily identify reactive causes for disease. On the other hand, only B and T cells could be identified using two tubes of the NHLS panel. However, the drawback for the NHLS tubes were that they could not allow for the complete identification of clonal T cells or NK cells. This inability to fully identify clonal T cells and NK cells resulted in the use of extra panel of markers. In cases where a T cell or NK cell malignancy were suspected an extended panel had to be used which would incur extra costs due to extra reagents, affecting turnaround time, with a resultant delay in releasing diagnostic results. The shortcomings of the NHLS panel are due to the use of 4 colour panels unlike the EuroFlow which made use of 8 colour panels and allowed for multiplexing of more antibody combinations.

Characterisation of CLL for both EuroFlow and NHLS was done after B-cell lineage had been identified. EuroFlow had only one tube with all the markers necessary for the identification of CLL whereas the NHLS panel employed all the 5 tubes in the panel to completely immunophenotype the disease. These differences were directly as a result of flow cytometry colours each panel and the respective instrument were able to handle. The 5 tubes of the NHLS panel were laborious to work with. The numerous tubes increase the possibility of human error during sample preparation in a routine diagnostic laboratory with a high rate of sample processing. A good example of such an error is when a technologist forgetting to add an antibody to one of the 5 tubes. In the EuroFlow panel, some antibody markers in the same tube shared the same colour channel. The markers that shared the same colour channels in the EuroFlow panel were CD56 and smlgk, CD8 and smlgλ, CD 20 and CD4 and, CD19 and TCRγδ. The markers in the same colour channels were for different cell lineage populations which ensured easy interpretation of results because the joined markers were not needed together during analysis. It is important to note that although the same colour channels were used by more than one marker, the EuroFlow panels are designed to ensure maximum staining of specific cell populations with optimal and non-conflicting antibody clones (van Dongen et al. 2012).

4.1.1.2 Instruments

The influence of the type instruments used in order to design a flow cytometry panel cannot be ignored. The NHLS Tygerberg use the BD FACSCaliber which is only
capable of detecting a maximum of 4 colours whereas the Beckman Coulter Navios can detect a maximum of 10 colours. The FACSCaliber in an NHLS laboratory is a critical tool that is effective at diagnostics immunophenotyping. However, compared to the EuroFlow panels, the NHLS flow cytometry antibody panel design was restricted by the flow cytometry instrument. If multicolour flow cytometry is to be successfully implemented for diagnostic purposes, it is imperative to acquire the right flow cytometry instrument. On the other hand, Flow cytometer instruments are relatively expensive and this poses a major challenge in our resource constrained settings.

4.1.1.3 Standard Operating Protocols

The standardised EuroFlow SOP’s for the diagnosis of CLL confer some advantages over the NHLS SOP’s in terms of data reproducibility. Although the NHLS SOPs are ‘standardised’ to a certain degree throughout the NHLS laboratories, variables such instrument type and the use of different antibodies in some laboratories hinders true standardisation. Implementing the EuroFlow SOP’S for routine practice would need validation and comparison in terms of the utility of the antibodies they suggest and of course the costs of using all their suggested markers. The drawback of the EuroFlow SOPs is that it takes away the freedom of individual laboratories to modify their SOPs. This may be a hard issue to tackle especially in SA where resource constraints are a major burden. While the EuroFlow SOP’s are effective, local laboratories do not necessarily have to adopt them. The NHLS flow cytometry panels are competent for their purpose, which is routine immunophenotyping. The drawback of each Institution in SA is the use of independent flow cytometry panels for diagnoses. This is the major reason behind difficulties in immunophenotype database creation as well as large-scale comparable immunophenotype research projects.

4.1.1.4 Antibodies usage and volumes

Due to resource constraints, the NHLS does centre based titrations of antibodies for optimal cost saving and don’t use the recommended volumes specified by the manufacturers. Although plausible, this practice adds to centre based variations as it
is largely dependent on expert interpretation which increases the possibility of user bias. To limit these variations, the NHLS routinely conducts external quality control (QC) assessments of its laboratories countrywide. The laboratories conduct inter-laboratory QC where possible and also subscribe to an international quality assurance programme such as the Royal Colleges of Australia (RCPA) quality assurance in flow cytometry. The RCPA sends a centralised specimen to interrogate and analyse using flow cytometry from each respective centre. The results are then evaluated by the RCPA and feedback given to the laboratory where there was failure with the view to correct the error. The errors could range from instrument setup through to software setup, processing, acquisition, and analysis. All NHLS laboratories also utilise standardised flow cytometry protocols and in this manner, the NHLS maintains a certain level of standardisation. However, where different centres utilise different antibodies and different clones of the antibodies on their flow cytometry panels, true standardisation cannot be achieved. Although the results would be effective for clinical diagnosis, the differences do not allow for direct immunophenotype comparisons, a critical need in flow cytometry research. In this regard, local laboratories including the NHLS could learn from EuroFlow and work towards standardisation of antibody panels.

With the above concerns considered, we recommend that the NHLS standardise their flow cytometry antibody panels for haematological malignancy immunophenotyping. Extensive research should be undertaken to ensure that the most appropriate yet cost effective antibody markers are included in the diagnostic panels. This can be facilitated by effective clone comparisons between antibodies (Figure 4.2) (Maecker et al. 2012), designing of cost effective cell population lineage screening tubes (van Dongen et al. 2012) and standardisation of data analysis protocols. Alternatively, the EuroFlow panels could be adopted for our population following proper validation of these panels within our setting.

Currently, the NHLS at TAH has in-house standardised instrument setup protocols including compensation. However, reagents used for these protocols should be standardised for complete uniformity of results. The biggest challenge in implementing multicolour flow cytometry would be the acquisition of new flow cytometers that can detect 8 colours as EuroFlow requires. Flow cytometers are expensive, and acquiring these instruments through the NHLS is a challenge due to the constant financial constraints despite the necessity for routine diagnosis. With the recent major scientific advancement in the pathogenesis of haematological malignancies the NHLS needs to
invest in instruments with higher sensitivities and specificities for diagnosis of malignancies.

With standardisation in place, NHLS comparable immunophenotype databases can be created. However, the recommendations for the NHLS will apply only within the South African borders and even so; they would be restricted within the NHLS string of laboratories. International and intercontinental database comparisons mean the adoption of standardised SOPs by either of the parties interested in joint research.

4.1.2 Establishment of Immunophenotypic database using Infinicyt™ flow cytometry software

Following confirmation of the 20 CLL cases via multicolour flow cytometry the cohort data were collated and a immunophenotype database consisting of locally flow cytometry CLL profiles was established using the Infinicyt flow cytometry analysis software. The database produced an immunophenotype map for the 20 CLL cases (Figure 3.5 B) showing the immunophenotype area where CLL cases in the TAH immunophenotype database fall within. Immunophenotype databases are electronic and can be easily accessed online for research purposes. An example is the recently privately published EuroFlow database (http://www.infinicyt.com). Standardised immunophenotype databases allow for the possibility of large scale multicentre immunophenotype studies. The possibility of worldwide comparable flow cytometry data is both exciting and crucial in furthering our understanding of human immunotype in diseased and healthy states. This might also serve as the stepping stone towards the increasingly advocated for human immunology project (HIPC) (Maecker et al. 2012). Success for the HIPC requires standardised immunophenotype data collection methods. Immunophenotype databases would make it possible for normal ranges of cell subsets and functions to be determined across variables such as age, gender, and ethnicity (Maecker et al. 2012). Currently, little is known of this important information.

EuroFlow offers partially automated diagnosis whereby immunophenotyping is done with respect to a reference database that is mathematically corrected thus eliminating the chance of user bias (van Dongen et al. 2012). It is however important to note that EuroFlow automated diagnostics cannot replace the expert but rather are an additional tool that aids in making a diagnosis at a faster turnaround time. In fact, expert users
are required to setup and grow the database. As the database progresses, less experienced users can also easily use flow cytometry with confirmation from the database stored immunophenotypes. In addition, the database goes a long way in assisting diagnosis of haematological malignancies in settings where flow cytometry experts are few and far between, such as low and middle-income countries.

To sum it up, an immunophenotype database is important because it;

- facilitates faster diagnosis by comparison of new cases with database cohort
- allows for clear identification of atypical cases that do not fit map’s ‘typical criteria’
- allows for easy manipulation of flow cytometry by less experienced users due to implementing partially automated data analysis
- statistically viable comparisons of populations from different geographical locations for example Africa and Europe
- provides easy storage for continuous long-term data collection

Thus, to have access to such a database would be of value. To our knowledge, the database we have developed is the first of its kind representing standardised CLL disease immunophenotype at TAH. The hope is to expand the development of such databases beyond CLL and to other NHLS centres and the wider South African regions in future.

4.1.3 Cost Analysis comparison between NHLS vs EuroFlow panels

The cost analysis for the CLL immunophenotype panels of the NHLS and EuroFlow showed that the EuroFlow was slightly cheaper. These calculations were based on the actual amounts of antibodies that were used for each of the two panels. This study has demonstrated that the EuroFlow multicolour flow cytometry panels have the advantages of improved cell type identification than the NHLS panels at a slightly lower cost. Although the cost difference is minute, its significance on a large scale national day to day use cannot be ignored.

4.1.4 Limitations of the multicolour flow cytometry study
Multicolour flow cytometry research is expensive and this forms part of our limiting factors which resulted in a small sample size. Statistically the minimum number of sample needed was 16 (Addendum 1). The second limitation was that although CLL is fairly uncommon, new patients that were naïve to treatment were also not always readily available due to the emotional factors associated with new cancer diagnosis.

Another limitation for the study was that we could not compare our database at TAH to that of the EuroFlow European population. This was because the EuroFlow database only became available online for a fee towards the end of our study. It launched their online European immunophenotype database for different haematological malignancy cohorts (“EuroFlow™ Databases Access” 2017). The database was built from over 20 collaborative laboratories across Europe. Large numbers of haematological malignancy samples from European patients were collected and analysed using their standardised flow cytometry (van Dongen et al. 2012). Without standardisation, this could not have been possible because of the flow cytometry variables from the collaborative laboratories. Thus, technical logistics could not allow for the comparisons to be made before the submission due date of this thesis. However, as a future directive the TLSG plans to make the comparisons in the near future.

4.1.5 Why lack of standardisation is a limitation in flow cytometry

The measurement of multiple parameters on individual cells using flow cytometry makes it possible to study cellular phenotype and function in the immune system. (Maecker et al. 2012). In this manner, the characteristics of many cell subsets can be mapped out from a complex mixture of suspensions for example blood (Maecker et al. 2012), lymph fluid and bone marrow.

In addition to surface cell markers, flow cytometry can also be used to assess intracellular components such as cytokines (Maecker & Maino 2004) and phosphoprotein (Perez et al. 2005)The use of immunophenotyping assays to identify lymphocyte, monocyte and/or granulocyte subsets is a crucial flow cytometry use (Maecker et al. 2012), particularly in the diagnosis of haematological malignancies.
Traditional methods of analysing flow cytometry data are prone to user error due to subjectivity. These traditional methods are reliant on expert interpretation which is subject to user bias. Currently, there is a lot of variation in the field of clinical and research flow cytometry.

Variations in flow cytometry involve but are not limited to instrument setup and sample preparation. In clinical flow cytometry, this is not necessarily a weakness as the goal is to correctly immunophenotype and aid diagnosis accordingly. However, these variations have major implications in research flow cytometry. The variations hinder progress because they limit comparability of multi-centre flow cytometry studies. This in turn limits progressive multicentre studies that cover large and different geographical regions.

Variables need to be carefully controlled in large studies that require inter-laboratory collaboration over large geographical regions (Maecker et al. 2012). Common variable in flow cytometry include reagents, sample preparation, instrument setup and data analysis (Figure 4.1) (Maecker et al. 2012). The variables were taken into consideration when the EuroFlow standardised methods were being developed (van Dongen et al. 2012).
4.1.6.1 Reagents

The diversity of antibody clones and fluorochromes can significantly influence results of flow cytometry studies (Figure 4.2) (Maecker et al. 2012). The differences may influence the reliable separation of positive and negative populations and also the percentage levels of positive cells due to poor separation. Furthermore, the poor separation may result in the mixture of dim and negative populations leading to an incorrect interpretation of results. Antibody specificity, clone and titre as well as fluorochrome in a staining cocktail should be carefully monitored for successful standardisation (Maecker et al. 2012).
Figure 4.2: Variability between the same antibody type with different clones. Extracted from Maecker et al. 2012.

4.1.6.2 Sample handling

Staining fresh whole blood or peripheral blood mononuclear cells (PBMC) immediately after draw is ideal but may not always be possible in large studies (Maecker et al. 2012). Cryopreservation of PBMCs may be a solution for storage of samples for future use but the depletion of certain cell types for example plasmablasts and dendritic cells is a major disadvantage; however, delays and shipment make PBMCs ideal (Maecker et al. 2012).

4.1.6.3 Instrument setup

Guided principles are important to govern the optimization of instrument settings to achieve standardisation. Instrument setup is mostly concerned with voltage gains applied to each fluorescence detector. These voltage gains influence the sensitivity of the detector to dim versus bright signals (Maecker et al. 2012). Differences in performances due to different instrument configurations from different manufacturers, especially multicolour flow cytometers, should be carefully monitored to achieve standardisation (Maecker et al. 2012). To overcome the instrument variations, target values are established across the different cytometers using
standardised control particles (Kalina et al. 2015). Voltage gains are set to place the
control particles (for example antibody capture beads) at a particular target channel
thereby achieving relatively similar instrument setup (Maecker et al. 2012).

4.1.6.4 Data Analysis

This forms one of the commonest variables in flow cytometry as well as the easiest
variables to address (Maecker et al. 2012). This is because re-analysis of existing data
is an option unlike the other stages of the flow cytometry experiment which cannot be
recovered nor corrected.
Central analysis is by one or few coordinated experts is a possible solution but can be
disadvantageous when dealing with large numbers of samples and multicentre studies
(Maecker et al. 2012). With this in mind, automated gating was developed and has
been shown to compete favourably with expert manual gating (Aghaeepour et al.
2013).

4.2 CLL Incidence study

For this part of the study, CLL incidence trends over a period of 6 years at TAH will be
described based on number of cases, age, gender, HIV, and molecular results. The 6-
year incidence of CLL cases at TAH between the years 2011 and 2016 were 80 in
total. Comparison of our results with the rest of SA could not be performed, due to: 1)
the unavailability of recent national population based cancer registries in SA, and 2)
the out-dated SA cancer registries do not report findings for specific cancer entities.
This highlighted the need for a radical improvement in the South African cancer
registries if it were to become an effective tool in the local management of cancers.

Population based cancer registries collect data on all new cases of cancer occurring
within a well-defined population. Previous studies have demonstrated the importance
of cancer registries in malignancy management (Parkin 2008; Mohammadzadeh et al.
2017). There is a critical need for updated national cancer records for SA. Lagging
national cancer registries form part of the reason behind limited descriptions of
leukaemia and lymphomas in SA. The records are paramount in monitoring cancer
disease incidence and crucial in overall management.
From as early as 1973, the importance of centralised cancer registries had already been identified (SEER Registries 2017) and currently, countries such as the USA have functional easily accessible online cancer registries that aid public health professionals in understanding and addressing the burden of cancer effectively (Center for Disease Control and Prevention USA 2017). The cancer registry data is effective for, but not limited to research purposes and national allocation of health resources in an efficient manner, a critical need in SA and Africa at large.

4.2.1 Six-year CLL incidence at TAH

In this study, the 6-year CLL incidence at TAH followed an irregular pattern as shown by the wide changes in the number of cases per year (Chapter 3, Figure 3.6). The disease generally presented at the same rate in men and women (Chapter 3, Table 3.4 and Figure 3.8) with no significant difference between the two genders (p>0.05). Our results differ from those of previous western studies that reported that CLL risk of occurrence as being slightly higher in men than in women (Diehl & Karnell 1999; Cancer Research UK 2017). In our setting, the results may be different due to the reluctance of men in seeking medical care (Addis & Mahalik 2003).

4.2.1.1 Age

The total mean and median age of CLL incidence at TAH was 67 years. This age is within the age range for CLL incidence in the USA and England (Howlader et al. 2016; Cancer Research UK 2017). Our data indicates that men present with CLL at a younger age than women (Figure 3.9). A significant difference between the age of CLL occurrence between men and women was noted (p value=0.024, 95% C.I) and our results agree with reports from other countries (Cancer Research UK 2017).

4.2.1.2 Molecular profiles

At TAH, different cytogenetic investigations for CLL patients were done on request by the haematologist. These cytogenetic tests during the period of our study were referred to the referral laboratory at GSH. The two methods of cytogenetic investigations employed at GSH are FISH and conventional karyotyping. FISH is mostly utilised due to its shorter TAT and specificity. It is generally recommended to assess for genetic
abnormalities in every CLL patient; however, due to cost factors, cytogenetic investigations for these patients are commonly done for those with aggressive or treatment resistant disease and sometimes for patients presenting with atypical CLL.

Nineteen patients were tested for the deletion 13q14 at TAH from 2011 to 2016. Literature denotes deletion 13q as the most common abnormality in CLL with over 50% incidence rates (Puiggros et al. 2014). Of the 19, only 6 patients (31%) were identified to have deletion 13q14. Although our study results are not close to 50%, deletion 13q abnormality at TAH had the highest incidence rate thus agreeing with literature compared to the rest of the other chromosomal abnormalities.

Trisomy 12 was also investigated in 19 patients and only 6 (26%) tested positive for this abnormality. The incidence rate at TAH is slightly higher than literature incidence rates of 15-20%. The prognostic characteristics of trisomy 12 are controversial but earlier reports suggested an intermediate risk associated with the abnormality (Döhner et al. 2000; Puiggros et al. 2014). Associations of trisomy 12 with other chromosomal abnormalities have been previously described (Döhner et al. 2000). At TAH, we observed that only one patient was double positive for both deletion 13q14 and trisomy 12 chromosomal aberration.

Interestingly, the one CLL patient with both trisomy 12 and deletion 11q22 had concurrent HIV. The patient was a male aged 51. The patient was refractory to CLL treatment and the disease was reported as progressive according to the follow up bone marrow reports. There was no evidence of 13q14 and deletion 17p aberrations in the patient. The second of the three patients with HIV was negative for both deletion 13q14, 17p13.1, and 11q22.3 as well as trisomy 12.

Deletion 11q22.3 was investigated in a total of 21 patients and 4 (19%) of these patients tested positive for the abnormality. The incidence agrees with literature where incidence ranges of 5-20% have been reported (Marasca et al. 2013; Döhner et al. 2000). Deletion 11q is associated with clinically progressive disease and patients often present with massive and multiple lymphadenopathy and generally a poor prognosis (H Döhner et al. 1997; Wierda et al. 2011). Unfortunately, we did not follow up our patients and therefore could not assess and compare this finding. The same cohorts of patients were also investigated for deletion of 17p13.1 and all of them tested negative for the chromosomal aberration.
Typical cases of CLL, based on morphology and immunotyping is a straightforward diagnosis and unlike other B-cells malignancies is generally not characterised by recurrent chromosomal translocations (Puiggros et al. 2014). However, atypical CLL often presents a diagnostic challenge because of its significant degree of resemblance morphologically to other aggressive B-cells malignancies. At TAH, chromosomal translocation investigations were performed in such cases to clearly differentiate between these entities and atypical CLL. Translocations t(8;14), t(11;14) and t(14;18) were investigated in 5 clinically suspected CLL patients using FISH. These investigations were probably requested due to an atypical morphology and therefore to exclude the possibility of other B-cells malignancies. None of the translocations were positive. Translocation t(8;14) is a confirmatory abnormality for Burkitt’s lymphoma. To exclude mantle cell lymphoma (MCL), its confirmative cytogenetic abnormality t(11;14) was investigated for. Follicular lymphoma (FL) was excluded from due to the negative t(14;18) abnormality, which is common in this malignancy entity.

Karyotyping was performed for 6 patients and 5 of these patients demonstrated normal male or female karyotype. However, 1 of the 5 patients had only 3 metaphases from peripheral blood tested. The possibility of an abnormal clone being present could not be completely excluded because a minimum of 10 metaphases is a requirement as the cut off for a significant investigation result for karyotyping at GSH. The remaining 1 CLL patient was the last of the 3 patients with concurrent HIV. This patient had no evidence of both the IGH/cyclin D1 (CCND1) fusion gene and the IGH/ B-cell lymphoma 2 (BCL2) fusion gene. Karyotyping for this patient was done in 20 metaphases from bone marrow culture. Of the 20, 5 cells contained Trisomy 12 and had lost the Y chromosome. However, 15 cells demonstrated normal male XY karyotype.
4.2.2 Limitations of Incidence study

The absence of entity specific and up to date South African cancer registries was a major limiting factor as it meant we could not compare our results to the rest of SA. The second limitation was; HIV data may have been under-represented because testing is usually performed at the clinics and hospital databases only have results for people tested on their site and not those coming for outside clinics.

4.2.3 Recommendations

Incomplete cancer registries are the major culprit for the wide knowledge gap in the incidence of CLL in SA. The last cancer national registry data was published half a decade ago; which would adversely affect strategic planning for cancer management and development of cancers treatments tailored for Africans. An update of registries is much needed in South Africa and Africa as a whole.

In SA, cancer registries do not account for specific cancers. With the advancements of cancer therapy to that of precision medicine, cancer management now needs to be tailored for each disease and patient. Based on these observations, we recommend that the South African cancer registries become more specific to different cancer entities. This would render the registries more usable and therefore directly impact on the planning for diagnosis and management of these cancers. This has already been implemented in the western world and has positively impacted on their management approach.

The influence of HIV on the South African health infrastructure due to its burden cannot be overlooked. Inconsistencies in the HIV results on the hospital patient registry online systems DISALAB and TrakCare are a cause for concern. 46% of CLL patients had no results available for the HIV tests. The inconsistencies using the DISALAB system was contributed by the fact that HIV testing is usually performed at a primary health facility. These factors should ideally not impact our data going forward as the TrakCare laboratory system allows visualising of results across the country.
4.3. Conclusions

Thus, to conclude, the present study emphasises the need to introduce more sensitive and specific diagnostic tools for routine haematological malignancy diagnoses. It creates an awareness for the need of standardisation of flow cytometry panels in diagnosis of haematological malignancies. Furthermore, sharing of data between laboratories would aid in creating a national database which further influences the approach to diagnosis and comparable results across the country. The validation of EuroFlow and comparison to our practise currently may result in new panels set up to suit our needs in South Africa and Africa, which might be more cost effective. Finally, we add knowledge to the incidence of CLL in SA over a 6-year period and hopefully this information will be used for a cancer registry.
Reference


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Addendum 1:

Preparation of wash buffer

Five hundred ml of wash buffer containing 350ml PBS of PBS (1%); 0.45g Sodium azide(NaN₃) (0.09%), an antibody preservative and 1g of BSA (0.2%) . BSA is a protein blocker whose prime function is to reduce non-specific binding of protein markers thereby reducing background and unclean data during flow cytometry. The mixture was gently swirled whilst avoiding the foaming of protein till the BSA dissolved. More PBS was added to reach a final volume of 500ml and gently mixed. The pH was checked and a pH of 7.3 was acceptable. The solution was then filtered through 0.2-micron sterilising filters (which brand) into sterile bottles and stored at 4°C.
Addendum 2

Sample Size Calculations

The following statistical formula was employed to determine the sample size (n);

\[ n = \frac{pq(z)^2}{d^2}, \text{ where;} \]

- \( n \) is sample size,
- \( p \) is sample proportion,
- \( q \) is \((1-p)\)
- \( z \) is level of significance
- \( d \) is margin of error

The sample size was calculated at 95% confidence interval \((z=1.96)\). The sample proportion was pegged at 99% as the study is dealing with NHLS confirmed CLL/SLL cases. A standard margin of error of 0.05 was used.

Therefore; \( n = (0.99 \times (1-0.99) \times (1.96^2))/0.05^2 = 15.212736 = 16 \) samples.

The minimum sample size needed for the study is 16.
Addendum 3

Hospital approval letter

ETHICS REFERENCE: S15/10/234

TITLE: Investigating the suitability of standardized Euroflow flow cytometry panels for the characterisation and diagnosis of chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) at Tygerberg Academic Hospital (TAH), South Africa.

Dear Mr F Musaigwa

PERMISION TO CONDUCT YOUR RESEARCH AT TYGERBERG HOSPITAL.

1. In accordance with the Provincial Research Policy and Tygerberg Hospital Notice No 40/2009, permission is hereby granted for you to conduct the above-mentioned research here at Tygerberg Hospital.

2. Researchers, in accessing Provincial health facilities, are expressing consent to provide the Department with an electronic copy of the final feedback within six months of completion of research. This can be submitted to the Provincial Research Co-Ordinator (Health.Research@westerncape.gov.za).

DR GG MARINUS
MANAGER: MEDICAL SERVICES [RESEARCH CO-ORDINATOR]

DR D ERASMUS
CHIEF EXECUTIVE OFFICE

Date: 26 June 2014
Administration Building, Francie van Zijl Avenue, Parow, 7500
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www.capegateway.gov.za
Addendum 4

Ethics letter

Approval Notice

Response to Modifications- (New Application)

04-Feb-2016

Musaigwa, Fungai F

Ethics Reference #: S15/10/234

Investigating the suitability of standardized Euroflow flow cytometry panels for the characterisation and diagnosis of chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) at Tygerberg Academic Hospital (TAH), South Africa.

Dear Mr Fungai Musaigwa,

The Response to Modifications - (New Application) received on 21-Jan-2016, was reviewed by members of Health Research Ethics Committee 2 via Expedited review procedures on 28-Jan-2016 and was approved.

Please note the following information about your approved research protocol:

Protocol Approval Period: 28-Jan-2016 - 27-Jan-2017

Please remember to use your protocol number (S15/10/234) on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:

Please note a template of the progress report is obtainable on www.sun.ac.za/rds and should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372

Institutional Review Board (IRB) Number: IRB0005239
The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

**Provincial and City of Cape Town Approval**

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Helene Visser at City Health (Helene.Visser@capetown.gov.za Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.

For standard HREC forms and documents please visit: www.sun.ac.za/hrs

If you have any questions or need further assistance, please contact the HREC office at 219389819.